

CONTROLS OF TACHYKININ RELEASE
IN THE MAMMALIAN SPINAL CORD.

CRICHTON WALKER LANG

DOCTOR of PHILOSOPHY
UNIVERSITY OF EDINBURGH
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For my mother and father.

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SUMMARY

The purpose of this series of investigations was to elucidate the endogenous control mechanisms active at primary afferent terminals of nociceptive origin in the dorsal laminae of the spinal cord grey matter. Such studies may enable the researcher to make certain conclusions about endogenous mechanisms of pain control in vivo. Furthermore, these studies may help to identify novel areas for the development of analgesic drugs or protocols of therapeutic value in both human and veterinary pain management.

Experiments were centred on one particular family of neuropeptides, the tachykinin peptides, and their release in response to peripheral noxious stimulation as determined by the antibody microprobe technique. A review of the anatomy and physiology of tachykinins in the spinal cord is presented at the start of this thesis. The antibody microprobe technique itself is also fully described.

Various means of modulating tachykinin release pharmacologically were tested and are presented in this thesis. The results presented here relate to studies on 1) morphine, 2) noradrenaline and the imidazoline derivative drug, medetomidine, 3) neuropeptide Y.

A short review on the pharmacological actions of each of these drugs is included. All the results presented are derived from experiments on barbiturate anaesthetised, spinalised cats.

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The work presented in this thesis is my own. All experiments were conducted by myself with assistance from the colleagues named above, with the exception of the results presented in section 5, the experiments for which being performed jointly with Prof.A.W.Duggan and Dr.P.J.Hope. Where results have already been published, this is acknowledged in the text. This thesis has not been submitted for the purposes of obtaining any degree or qualification from any other academic institution.

PUBLICATIONS.

The following publications were all derived from experimental work with which I was involved during my period of study.

Hope, P.J., **Lang, C.W.** and Duggan, A.W., Persistence of immunoreactive neurokinins in the dorsal horn of barbiturate anaesthetized and spinal cats, following release by tibial nerve stimulation, *Neurosci. Lett.*, 118 (1990) p25-28.

Duggan, A.W., Hope, P.J. and **Lang, C.W.**, Microinjection of neuropeptide Y into the superficial dorsal horn reduces stimulus evoked release of immunoreactive substance P in the anaesthetized cat, *Neuroscience*, 44 (1991) p733-740.

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Duggan, A.W., Schaible, H-G., Hope, P.J. and **Lang, C.W.**, Effect of peptidase inhibition on the pattern of intraspinally released immunoreactive substance P detected with antibody microprobes, *Brain Res.*, 579 (1992) p261-269.

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Lang, C.W., Hope, P.J., Grubb, B.D. and Duggan, A.W., Microinjection of noradrenaline or medetomidine does not alter the pattern of nerve stimulus-evoked release of substance P in the spinal cord of the anaesthetised cat: a study with antibody microprobes, *Br. J. Pharmacol.*, 112 (1994) p951-957.

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ABBREVIATIONS.

The following is a complete list of all abbreviations used at points throughout the following text, figures and figure legends.

5HT	serotonin
ACE	angiotensin converting enzyme
APTES	aminopropyltriethoxysilane
BSA	bovine serum albumin
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CNS	central nervous system
Cont.	control(s)
cpm	counts per minute
EPSP	excitatory post-synaptic potential
GABA	gamma-aminobutyric acid
HPLC	high pressure liquid chromatography
-LI	-like immunoreactivity eg. SPLI
ir	immunoreactive
mRNA	messenger RNA
MED	medetomidine
NA	noradrenaline
NAIBS	non-adrenergic imidazoline binding site
NPY	neuropeptide Y
NK	neurokinin
NKA	neurokinin A
NKB	neurokinin B
NPK	neuropeptide K
NPγ	neuropeptide gamma
NA	noradrenaline
PBS	phosphate buffered saline
PPT	preprotachykinin
PNS	peripheral nervous system
RER	rough endoplasmic reticulum
SEM	standard error of mean
SG	substantia gelatinosa
SP	substance P
Stim.	stimulus

SECTION 1
TACHYKININ BIOCHEMISTRY, PHYSIOLOGY
AND PHARMACOLOGY IN MAMMALIAN SYSTEMS

1.1 A BRIEF HISTORY OF TACHYKININS.

In 1931, while testing alcohol extracts of various equine tissues for acetylcholine activity, von Euler and Gaddum (1931) noted that some extracts, particularly those taken from intestine and brain, showed hypotensive and gut spasmogenic actions. This activity could not be attributed to choline or acetylcholine as it was not altered to any significant extent by the application of atropine. The active component was named substance P (P for 'preparation'). Although this was the first reference to a tachykinin peptide, further advances in the study of tachykinins were slow, as complete purification of substance P (SP) proved difficult to achieve.

Indeed it was a non-mammalian tachykinin, eledoisin, taken from the Mediterranean octopus *Eledone moschata* which was the first to be successfully purified and sequenced (Erspamer and Anastasi, 1962a,b). Eledoisin and SP extracts were shown to share many pharmacological activities and protease sensitivities, but they could also be readily distinguished by their respective potencies in a variety of mammalian systems including rabbit large intestine, guinea-pig ileum and dog cardiovascular preparations. By 1971 two amphibian tachykinins, physalaemin and phyllomedusin, had also been isolated (Erspamer, 1971). As the physiological actions of all members of this peptide group were notably faster than bradykinin and bradykinin-like peptides, especially on gut preparations, it was at this point that the term '**tachy**-kinin' was adopted to describe this family of peptides.

A few years prior to this establishment of tachykinins as a peptide group in their own right, SP had been rediscovered in

extracts of bovine and rat hypothalamic tissue (Leeman and Hammerschlag, 1967). The, as then, unidentified peptide was shown to stimulate salivation in rats, and this activity was not blocked by cholinergic or adrenergic drugs. Within a few years this sialogogic peptide had been purified (Chang and Leeman, 1970), and identified as being the same peptide as that named by von Euler and Gaddum in 1931. Forty years after its initial discovery SP was finally sequenced and synthesised (Chang et al, 1971, Tregear et al, 1971).

Other non-mammalian tachykinins continued to be isolated and sequenced: uperolein (Anastasi et al, 1975), kassinin (Anastasi et al, 1977), pro-kassinin and hyalambatin (Yasuhara et al, 1981). The existence of these non-mammalian peptides proved to be of less interest, however, than the search for more mammalian peptides sharing the now well established canonical carboxyl-terminal amino acid sequence for tachykinins; **-Phe-X-Gly-Leu-Met-NH₂** where **X** is either an aromatic (**Phe, Tyr**) or branched aliphatic (**Val, Ile**) amino acid residue.

It was proposed (Erspamer, 1981) that the amino acid sequence of SP was older, in evolutionary terms, than those of the non-mammalian tachykinins. Erspamer also suggested that the then current conviction that SP was the only mammalian tachykinin and that the different tachykinins were subject to phyllogenetic barriers with regard to their distribution would eventually be proved incorrect. Of great significance (as we shall later see), Erspamer also pointed out that the developing techniques of immunocytochemistry and radioimmunoassay were only valid in the study of SP if highly specific antisera were used.

In 1983 as a result of testing porcine spinal cord extracts for a tachykinin-like effect on guinea-pig ileum contractility, a novel mammalian tachykinin, named neuromedin K, was isolated and sequenced (Kangawa et al, 1983). Subsequently the same technique ~~revealed~~ ^{revealed} a second new tachykinin, named neuromedin L (Minamino et al, 1984). At the same time other groups were also successful in isolating what proved to be the same peptides, resulting in these two new mammalian tachykinins being given several different names (Kimura et al, 1983, Maggio et al, 1983, Nawa et al, 1984). Attempts have been made to standardise the naming of these two tachykinins, neurokinin A (NKA) and neurokinin B (NKB) now being the most favoured names.

NKA = substance K, neurokinin alpha, neuromedin L.

NKB = neurokinin beta, neuromedin K.

In 1985 an N-terminally extended form of NKA was isolated from porcine brain extracts (Tatemoto et al, 1985), which showed enough biological activity to preclude it from being merely a precursor form of NKA. This tachykinin was named neuropeptide K (NPK). More recently a fifth mammalian tachykinin, neuropeptide gamma (NPγ), was isolated from rabbit intestine (Dam et al, 1990b, Kage et al, 1988). The amino-acid sequences of these five mammalian tachykinins are illustrated in **figure 1**.

Despite these recent additions to the tachykinin peptide family (new non-mammalian tachykinins also continue to be identified), most work to date has focused on the 'oldest' member of the group, SP, and to a lesser extent on NKA and NKB. The numerous review articles dealing with tachykinin structure, synthesis and functions (Duggan

FIGURE 1. Amino acid sequences of the five established mammalian tachykinin peptides; substance P, neurokinin A, neurokinin B, neuropeptide K, and neuropeptide gamma (γ). The highly conserved canonical C-terminal amino acid sequence is underlined. Note also that neuropeptide K is simply an N-terminally extended form of neurokinin A.

FIGURE 1.
AMINO ACID SEQUENCES OF MAMMALIAN TACHYKININS.

<u>Substance P</u>	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH ₂
<u>Neurokinin A</u>	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂
<u>Neuropeptide K</u>	Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu- Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His- Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂
<u>Neurokinin B</u>	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH ₂
<u>Neuropeptide γ</u>	Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg- His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂

and Weihe, 1991, Erspamer, 1971, Helke et al, 1990, Maggio, 1988, Nakanishi, 1991, Nicoll et al, 1980, Otsuka and Yoshioka, 1993, Pernow, 1953,1983, Regoli et al, 1990, Wilcox, 1991) continue to become incomplete as the volume of work and the number of techniques employed to study these peptides increases.

1.2 MOLECULAR BIOLOGY OF TACHYKININS

The stages involved in the neuronal synthesis of tachykinins and of other groups of neuropeptides, from gene transcription to post-translational processing are now well understood. Unlike classical neurotransmitters such as noradrenaline, which are synthesised in nerve terminals by a variety of enzymatic reactions, neuropeptides are initially synthesised in ribosomes, within the neuronal cell body, as larger precursor forms within which the neuropeptide resides. The translation of genetic information in the cell nucleus into messenger ribonucleic acid (mRNA), and the translation of that mRNA, within ribosomes, into precursor proteins follows standard peptide synthesis mechanisms (see Sherman et al, 1989) and will not be discussed further here. Beyond this stage, however, notable specialisations in neuropeptide synthesis exist. The first product of mRNA transcription is a **pre-propeptide**, which contains a signal peptide facilitating movement of the precursor molecule into the rough endoplasmic reticulum (RER). Within the RER the signal peptide is cleaved off the molecule to form a **propeptide**, which is then transported to the Golgi apparatus where it is packaged into granules and vesicles before transport to the nerve terminal. Various enzymes

are also contained in these granules and these act to 'release' the neuropeptide from the propeptide molecule and further modify it as the granule passes from cell body to nerve terminal. These final stages in neuropeptide synthesis (~~where~~^{which} are discussed more fully at a later point) are collectively termed "post-translational processing".

The above overview (illustrated in **figure 2**) applies generally to neuropeptides, of which the tachykinin family form only a small proportion. This pattern applies to pro-dynorphin, pro-enkephalin and pro-opiomelanocortin related peptides, and also to pituitary peptides, releasing factors and release-inhibiting factors. More (or less) information is available about the biosynthesis of each specific group, and a more detailed consideration of tachykinin synthesis follows.

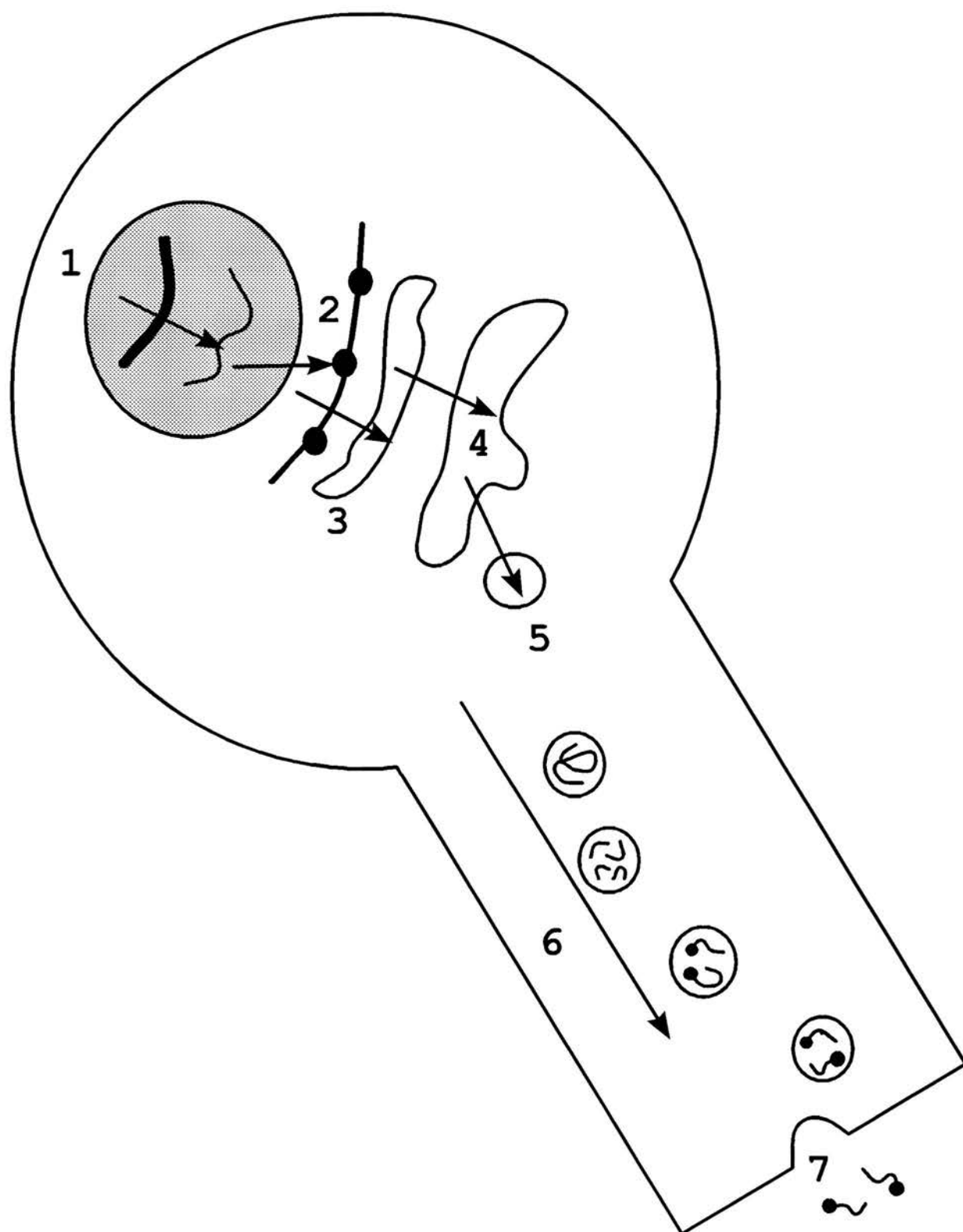
Two genes are currently considered to be involved in the coding of tachykinin precursor molecules. The **pre-protachykinin (PPT) I** or **A** gene contains the nucleotide sequences responsible for the synthesis of SP, NKA, NPK and NPY precursor peptides (Nawa et al, 1983). A separate gene, the **PPT II** or **B** gene, encodes the mRNA responsible for the production of NKB precursor peptide (Kotani et al, 1986). Although the PPTB gene is very similar to the PPTA gene in terms of its DNA sequence it has not yet been shown to encode any other tachykinin precursor molecules.

FIGURE 2. An overview of the cellular pathways involved in peptide synthesis, as applicable to tachykinin neuropeptides. The figure represents a highly simplified neurone with cell body and short axon.

- 1) DNA is transcribed in the nucleus to form messenger RNA
- 2) Messenger RNA is translated by ribosomes on the surface of the rough endoplasmic reticulum (RER) to form a pre-propeptide; this incorporates a signal protein which facilitates the movement of the pre-propeptide into the lumen of the RER itself.
- 3) Inside the RER the signal protein is cleaved off to form a propeptide molecule.
- 4) The propeptide is transported to the Golgi apparatus where it is packaged into granules and vesicles.
- 5) Vesicles are released into the cell body and are transported to the nerve terminal by axoplasmic transport.
- 6) During this axoplasmic transport, various enzymes (also contained in the packaged granules) act on the propeptide, converting it into the final tachykinin sequence. This is termed post translational processing.
- 7) Following an appropriate stimulus, vesicles filled with the now functional peptide release their contents into the synaptic cleft by exocytosis.

FIGURE 2

OVERVIEW OF NEUROPEPTIDE SYNTHESIS



It was initially shown that two (Nawa et al, 1983) and then that three (Krause et al, 1987) pre-protachykinin mRNAs could be produced from the PPTA gene by alternative splicing of the individual exons of the gene:

alpha PPT mRNA from exons 1,2,3,4,5 and 7

beta PPT mRNA from exons 1,2,3,4,5,6 and 7

gamma PPT mRNA from exons 1,2,3,5,6 and 7

Thus the differences between these three mRNAs involve only exons 4,5 and 6 of the PPTA gene. (See also **figure 3**). How alternative splicing of the transcripts of these exons is controlled is not known. The different proportions of the three mRNAs seen not only between species but also between different tissues within one species (Kimura et al, 1984, Krause et al, 1987) do, however, suggest that some form of control may exist at this level. For example, the ratios of alpha:beta PPT mRNA in rat and bovine central nervous system (CNS) tissue are <1:20 and 3:1 respectively, despite marked homology in the structure of the PPTA gene between the species. In the bovine, although alpha PPT mRNA was the predominant mRNA form in CNS tissues, levels of beta PPT mRNA were greater in gut and thyroid tissues (Carter and Krause, 1990).

Although the proportions of alpha, beta and gamma PPT mRNAs have not been demonstrably altered by any exogenous factor, numerous factors do seem to alter both PPTA gene expression and the overall rate of tachykinin synthesis. Dopamine agonists (stimulatory) and antagonists (inhibitory) have been shown to have such an effect in rat striatum (Bannon et al, 1987, Haverstick and Bannon, 1989, Lindefors et al, 1989) suggesting that interactions with other

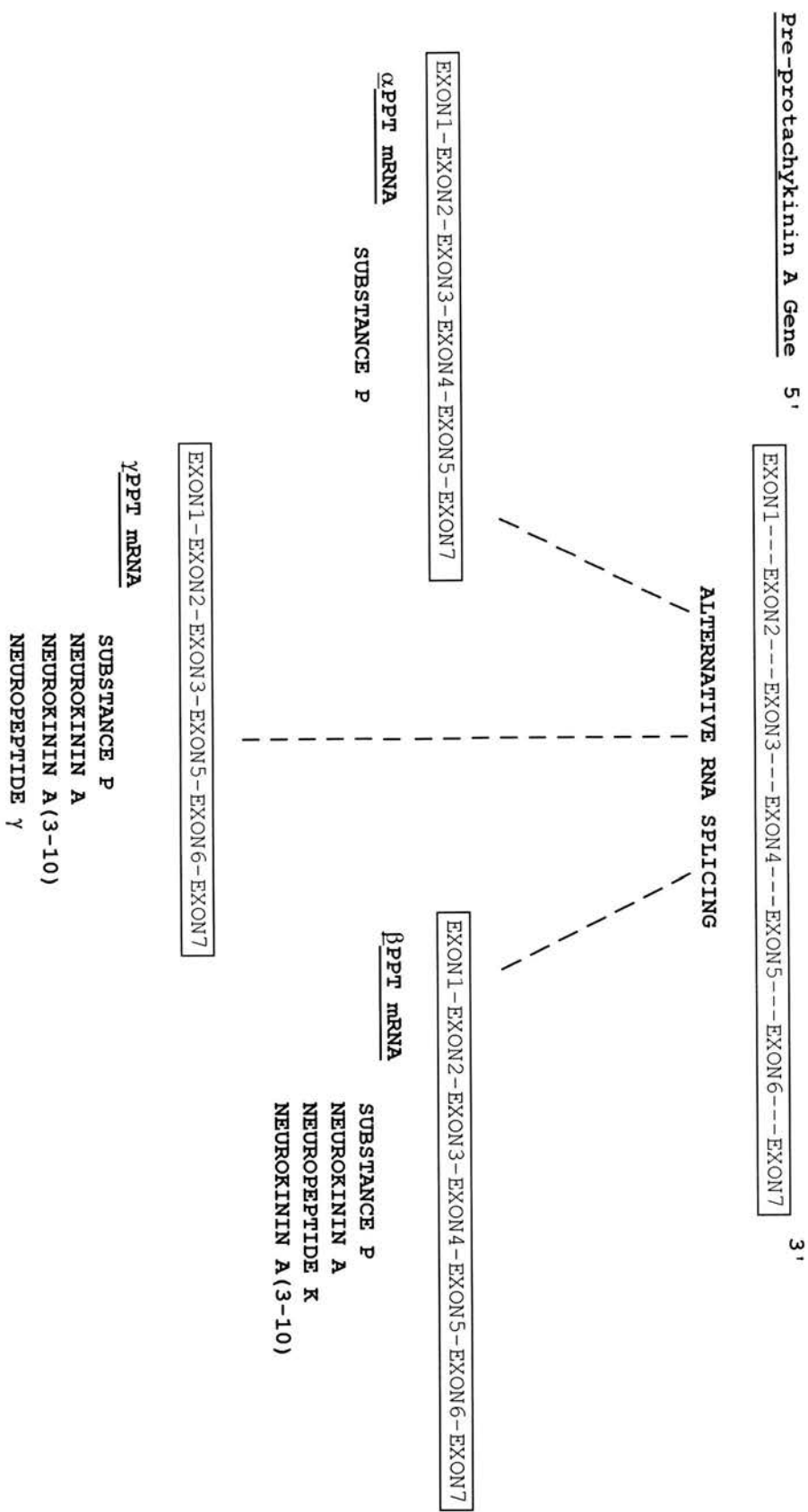
intrinsic messenger systems may account for variations in tachykinin gene expression. Roles for nerve growth factor (stimulatory) in cultured dorsal root ganglion cells (Lindsay et al, 1989) and oestrogen in the hypothalamus (stimulatory) and pituitary (inhibitory) (Brown et al, 1990) have also been suggested.

The pre-protachykinins are large peptides (alpha PPTA = 112 amino acids, beta PPTA = 130, gamma PPTA = 115, PPTB = 126), certainly when compared to the tachykinins themselves (SP = 11 amino acids, NKA = 10, NPK = 36, NPY = 21, NKB = 10). It seems likely that several enzymes may be responsible for the post-translational processing of the pre-protachykinin molecules. Sites which could be utilised by both endoproteases and aminopeptidases have been identified (Krause et al, 1987, MacDonald et al, 1989), and petidyl-glycine- α -amidating mono-oxygenase (PAM) has been proposed as the enzyme responsible for the C-terminal amidation of SP and NKA (Graham and Gallop, 1989).

Antibodies raised to N-terminally extended tachykinins and to tachykinin fragments have been used to identify these forms in nervous tissue (Kream et al, 1985). Whether or not these peptides represent novel tachykinins, as in the case of NPK, or simply intermediates in post translational processing and metabolites produced during tachykinin breakdown is open to question. The status of one such tachykinin fragment, NKA (3-10), which could be produced from both beta and gamma pre-protachykinin, is currently under debate.

FIGURE 3. Alternative splicing of the PPTA gene results in the formation of three different preprotachykinin messenger RNAs (α , β and γ mRNA), depending on the sequence of exons included during DNA transcription. Each type of mRNA has the potential to encode a different range of tachykinin peptides, as illustrated.

FIGURE 3
ALTERNATIVE RNA SPLICING OF THE PPTA GENE.



1.3. TACHYKININ DISTRIBUTION.

I. PERIPHERAL AND CNS DISTRIBUTION

The information presented in this section is summarised in **table 1**.

Prior to the purification and sequencing of SP in the early 1970's, distribution studies on SP relied simply on its extraction from the various body tissues, either by alcohol extraction (von Euler and Gaddum, 1931,) or a salting out procedure with ammonium sulphate (Pernow, 1953). These extracts were then tested either for a stimulating effect on isolated smooth-muscle preparations such as guinea-pig ileum or rabbit jejunum, or alternatively for their hypotensive effect in the rabbit. von Euler and Gaddum (1931) had noted in the first report of SP that, in the horse, its activity was greatest in alcohol extracts of intestine or whole brain. Pernow (1953) performed an exhaustive study of SP distribution in various tissues from several species, (a few more recent studies on tachykinin distribution in peripheral tissues might also be noted, Brodin and Nilsson, 1981, Bucsecs et al, 1983, Ekstrom et al, 1988). In the horse, dog, guinea-pig and rat, SP was shown to be present in the jejunum, although other areas of the gastrointestinal tract were not studied in these species. In the monkey, cow, sheep, pig and cat all areas of the digestive tract, including the stomach and oesophagus, were shown to contain SP with the levels being generally greatest in the jejunum and ileum. In the dog many other tissues were also studied. SP activity was identified in all areas of the CNS examined, in the spinal cord (grey and white matter combined) and in a variety of peripheral ganglia and nerves, where a high level of SP

activity was noted in the dorsal roots. The urinary bladder, ureters, non-gravid uterus, gall bladder, diaphragmatic skeletal muscle, lungs, heart, liver, spleen, kidneys, pancreas, thyroid and parotid salivary glands were all shown to contain SP, albeit in much smaller amounts than that found in nervous tissue.

The extracts obtained in these early studies were almost certainly impure, and much of the activity attributed to SP is likely to have been due, in part at least, to the presence of other tachykinin peptides or unrelated compounds. Although this by no means makes the findings of such studies invalid, care must be taken not to overinterpret the results. The other clear limitation of simple extraction/bioassay investigations is the inability to identify, at a cellular level, which cell types and cell structures contain tachykinins. After the sequencing of SP (and later of the other tachykinins) a more detailed understanding of tachykinin distribution could be achieved using radioimmunological and immunohistological techniques. A vast amount of work has been carried out with these techniques, and discussion of such studies will be limited principally to those involving the nervous system, in keeping with the topic of this thesis.

SP-like immunoreactivity (SPLI) in the brainstem has been extensively mapped in the rat (Brownstein et al, 1976, Douglas et al, 1982, Kanazawa et al, 1984, Kanazawa and Jessell, 1976), cat (Lovick and Hunt, 1983) and in man (Cooper et al, 1981, Emson et al, 1980, Gale et al, 1978). SPLI is present in a large number of sites within the brainstem, with notably high levels seen in the caudate nucleus and the zona reticularis of the substantia nigra.

Early immunohistochemical studies revealed the presence of SPLI in 20-25% of cell bodies in rat spinal and trigeminal ganglia, and in nerve fibres within rat, cat and human spinal cord (Cuello et al, 1976, Hokfelt et al, 1975a,b, Nilsson et al, 1974). Bioassays with, now purer, SP extracts had identified high levels of SP-like activity in bovine dorsal-root tissue (Takahashi et al, 1974), suggesting a link between SP and sensory systems. Later studies, in rat, cat, primate and man, confirmed this and further suggested that SP was particularly associated with smaller diameter C- or A-delta type sensory neurones carrying inputs from peripheral nociceptors. (Barber et al, 1978, Bisby and Keen, 1986, Chan-Palay and Palay, 1977a,b, Cuello and Kanazawa, 1978, Difiglia et al, 1982, Gibson et al, 1988, Hokfelt et al, 1977, Hunt et al, 1981, Kawatani et al, 1985, Lamotte and Shapiro, 1991, Ljungdhal et al, 1978, Maggio and Hunter, 1984, Pickel et al, 1977, Villar et al, 1991). SPLI within the spinal cord is associated with neuronal cell bodies (intrinsic neurones) only in the dorsal horn of the grey matter. SPLI-containing fibres are found throughout the whole of the grey matter and occasionally extending into the white matter. The density of these SPLI fibres is, however, greatest in laminae I and II of the superficial dorsal horn, and around the central canal, the major site of somatic and visceral primary afferent fibre termination (Kuo et al, 1983, Kuo and de Groat, 1985, Light and Perl, 1977, Rethelyi et al, 1979). In the dorsal horn such fibres are seen to make axodendritic contacts with intrinsic dorsal horn neurones.

It must be emphasised that not all SPLI in the dorsal spinal cord is associated with primary afferent terminals, as much as 40-50% may be associated with intrinsic neurones of the spinal cord or with

bulbo-spinal projections (Ljungdhal et al, 1978). Moderately high numbers of fibres showing SPLI are seen in lamina V of the dorsal horn. The localisation of SPLI to dense-cored vesicles within synaptic terminals has also been described (Cuello et al, 1977, Cuello and Kanazawa, 1978, Merighi et al, 1989, Pelletier et al, 1977).

Larger peripheral nerves such as the vagus nerve show SPLI in approximately 10% of their fibres, and again these seem to be predominantly smaller unmyelinated neurones (Gamse et al, 1979b). The detection of SP in peripheral tissues can largely be accounted for by its presence in nerve fibres within those tissues. For example, the gastrointestinal tracts of various species have an extensive population of intrinsic neurones showing SPLI (Franco et al, 1979, Malmfors et al, 1981, Nilsson et al, 1975a, Pearse and Polak, 1975), and also an extrinsic neuronal supply involving fibres showing SPLI (Costa et al, 1980, Hayashi et al, 1982, Lundberg et al, 1978). In peripheral tissues these SP immunoreactive terminals may be associated with epithelial or secretory components, but are also frequently seen to contact the walls of blood vessels (Anggard et al, 1979, Brodin et al, 1981, Dalsgaard et al, 1983, Ichikawa et al, 1990). Free SP immunoreactive material can also be detected in blood (Gamse et al, 1978, Nilsson et al, 1975b, Yanaihara et al, 1976) and this may derive from the SP innervation of vascular beds in peripheral tissues such as the gut (Gamse et al, 1978). Occasionally, for example in tooth pulp, free nerve terminals showing SPLI are identified (Brodin et al, 1981).

In the majority of the above studies the antibodies used were polyclonally derived and directed at the C-terminal sequence of SP.

Although SP is the most abundant tachykinin in mammalian tissues (Arai and Emson, 1986), all currently identified members of the mammalian tachykinin peptide family share a common C-terminal sequence. Care must then be taken in immunochemical studies to avoid using antisera with a high degree of cross-reactivity between two or more members of the tachykinin family. The possibility exists that, prior to the discovery of neurokinin A in 1983, some of the antisera used were not fully specific for SP, and the distributions of SPLI reported represent those of the tachykinin group as a whole rather than just that of SP. It might be noted that although the combination of high pressure liquid chromatography (HPLC) with radioimmunoassay has been of great value in the detection and identification of tachykinins (Aria and Emson, 1986, Lee, J-M. et al, 1986, Ogawa et al, 1985), such precise chemical identification cannot be directly incorporated into immunocytochemical studies, which give a much more detailed picture of peptide distribution. In more recent years, however, antibodies showing very low cross-reactivities between tachykinins or raised not against the peptides themselves but to the PPTA or PPTB mRNAs have allowed yet further clarification of the patterns of tachykinin distribution.

The overall distribution of SP, NKA, NKB and NPK throughout the various tissues of the mammalian body appears to be similar (Arai and Emson, 1986, Valentino et al, 1986). The proportions of the different tachykinins does, however, vary from tissue to tissue (Brodin et al, 1986, Nagashima et al, 1989, Ogawa et al, 1985, Takano et al, 1988, Tateishi et al, 1989, 1990) suggesting the existence of some mechanism or mechanisms regulating alternative gene splicing and/or post-translational processing as outlined in the previous chapter.

Hybridisation histochemistry studies, using ~~antibodies~~ ^{digoxigenin ribonucleotide probes} raised against PPTA mRNA or PPTB mRNA, have shown that in some CNS areas such as the rat habendula or caudate putamen (Burgunder and Young, 1989a,b) although both PPT genes are present throughout the area, individual cells express only one of the two genes. In other areas, such as the raphe nuclei and the nucleus of the lateral olfactory tract all cells express one identical gene type, PPTA and PPTB respectively (Warden and Young, 1988). (It must be noted that the presence of PPT mRNA within a neurone may not be equivocal with the release of tachykinins from the terminals of that neurone).

Some observations may have significance in elaborating the functional roles of the different members of the tachykinin family in the spinal processing of nociceptive inputs. The distribution of immunoreactive (ir)SP within the spinal cord has already been described; a low density of immunoreactive fibres throughout the spinal cord with a much higher density seen in laminae I and II of the superficial dorsal horn, the main area of termination for primary afferent fibres and in lamina X surrounding the central canal. The distribution of PPTA mRNA in the spinal cord is similar to that seen for irSP, and PPTA mRNA is also found in sensory neurones of dorsal root ganglia. Furthermore, PPT-derived mRNAs are elevated in the spinal cord following maintained, noxious, peripheral insult (Minami et al, 1989, Noguchi and Ruda, 1992).

PPTB mRNA containing cells predominate in lamina III of the dorsal grey matter and appear to be absent from dorsal root ganglion cells (Warden and Young, 1988). Studies with a selective antibody for NKB confirmed its preferential location to lamina III of the dorsal spinal cord, and also showed that irNKB levels within the spinal cord

were unaffected by dorsal rhizotomy, whereas this procedure led to a marked drop in spinal cord irSP (Ogawa et al, 1985). Collectively this data suggests a close association between the PPTA related tachykinins; SP, NKA and possibly also NPK and NPY , and primary afferent transmission in the dorsal spinal cord. This association does not appear to exist for the PPTB related member off the tachykinin family, NKB.

In peripheral tissues also, recent studies have suggested that some sites, such as the guinea-pig ileum and urinary bladder, which stain for irNKA and irSP, do not immunoreact with an NKB-specific antiserum (Too et al, 1989a,b). Clearly, despite their marked structural similarities, the products of the two tachykinin encoding genes do have different distributions, although these distributions overlap in some areas, particularly in the CNS. Functional differences may, therefore, also exist.

A small number of studies have investigated tachykinin distribution in non-mammalian species; chicken spinal cord (Sakamoto and Atsumi, 1989), chicken spinal ganglia (Schultzberg et al, 1978), lamprey CNS (van Dongen et al, 1986). As in mammals a close association between irSP and sensory transmission in the spinal cord is observed, and irSP staining is again heaviest in the superficial dorsal horn.

The ontogeny of tachykinin expression in cat and rat CNS has also been studied (Boylan et al, 1990, Diez-Guerra et al, 1989). The results of these studies indicate that tachykinin expression in the CNS of these species develops not only throughout gestation but also continues for several weeks after birth. This may have significance in the interpretation of some of the studies on tachykinin release

and function which employ a preparation of neonatal spinal cord such as that of Otsuka and Konishi (1976). The findings of such studies will be discussed in a later section. A requirement for nerve growth factor in the development of tachykinin-containing sensory nerve fibres has been reported (Mayer et al, 1982, Otten et al, 1980).

1.4 TACHYKININ DISTRIBUTION.

II. COEXISTENCE BETWEEN TACHYKININS OR WITH UNRELATED PEPTIDES IN THE CENTRAL NERVOUS SYSTEM.

Immunocytochemical studies employing N-terminally directed (and therefore more selective) antisera to individual tachykinins have identified populations of primary afferent fibres staining for SP alone, SP and either NKA or NPK or SP, NKA and NPK. (Dalsgaard et al, 1985, Ogawa et al, 1985, rat). These variations may relate to the three different mRNAs which can be derived from the PPTA gene (see earlier section) each of which has the ability to encode a different combination of tachykinin peptides.

Use of multiple antibodies can also detect coexistence with other classes of neurotransmitter within individual neurones, and this topic was reviewed by Dalsgaard, 1988. The specificity of antisera used in these studies, however, generally precludes the differentiation of the individual tachykinin(s) involved. Thus in afferent fibres tachykinins are seen in 50-100% of neurones showing immunoreactivity for calcitonin gene-related peptide (CGRP), representing neuronal input derived from all peripheral tissues (Sharkey et al, 1989, rat). Some of the afferents from cutaneous

receptors additionally stain for cholecystokinin (CCK) and prodynorphin, whilst afferents arising from skeletal muscle vasculature contain tachykinins, CGRP and CCK (Gibbins et al, 1987, guinea-pig). Somatostatin coexists with tachykinins and CGRP in somatic afferent fibres and a sub-population of these fibres also contains galanin, but the specific origin of such fibres has not been determined (Garry et al, 1989, cat). The excitatory amino acid, glutamate, has been shown to coexist with SP in primary afferent structures of the spinal cord (Battaglia and Rustioni, 1988, rat and primate, de Biasi and Rustioni, 1988, rat). Other peptides, such as endothelin (Giad et al, 1989, man), enkephalin-like peptides (Senba et al, 1989, rat) and mammalian bombesin-like peptides (Cameron et al, 1988, cat) have also been demonstrated to coexist with tachykinins in primary sensory structures. As for other CNS sites, acetylcholine in the pons of the rat (Vincent et al, 1983) and serotonin (5HT) in the medulla of both rat and cat (Lovick and Hunt, 1983) have been shown to coexist with tachykinins.

Overall the pattern of neurotransmitter/peptide coexistence in the CNS is being increasingly revealed as extremely complex in nature. The functional significance of peptide multiplicity within individual neuronal structures is still unclear, but an interesting interaction between CGRP and SP has recently been reported (Schaible et al, 1992). Microinjection of CGRP into the superficial dorsal horn of the cat was shown to facilitate the spread of SP away from sites of release in the same area. The altered pattern of irSP detection following CGRP application was almost identical to that seen following antagonism of the peptidases responsible for SP degradation in vivo (Duggan et al, 1992, as illustrated in **figure 10**, section 2),

suggesting that following co-release of SP and CGRP the role of CGRP might be to block such metabolism of SP, thus altering the area of spinal cord over which it could have an effect on neuronal structures. The potentiation of SP mediated effects at the spinal level by CGRP in the rat has also been reported in electrophysiological (Biella et al, 1991, Woolf and Wiesenfeld-Hallin, 1986) and behavioural studies (Oku et al, 1987, Wiesenfeld-Hallin et al, 1984). Thus interactions between co-released compounds may not be confined to their direct effects on pre- or post-synaptic elements.

1.5 TACHYKININ RECEPTORS.

Observations that the rank order of potencies for SP and non-mammalian tachykinins, or derivatives of these compounds, varied quite subtly in various bioassays utilising mammalian tissue (Buck et al, 1984, Hunter and Maggio, 1984, Lee, C-M. et al, 1982, Maggi et al, 1986, Piercey et al, 1985, Rosell et al, 1983, Szeli et al, 1977, Watson et al, 1983) led to the suggestion that subtypes of mammalian tachykinin receptor might exist. The specificity of receptor subtypes for the different tachykinins was thought to relate to the N-terminal amino acid sequences of the different peptides. The general range of biological activities exhibited by the tachykinins seemed to be dependant on the integrity of the common C-terminal amino acid sequence, and also related to whether the residue **X** in the C-terminal sequence **PHE-X-GLY-LEU-MET-NH₂** was an aromatic or aliphatic amino acid. Substitution of single N-terminal amino acid residues on the other hand could have only slight effects on biological activity,

TABLE 1. Distribution of tachykinins in selected areas of the central nervous system and selected peripheral tissues. Levels of tachykinins from different studies have been standardised to pmol g⁻¹ tissue. Data is derived from references cited in text.

TABLE 1.

DISTRIBUTION OF TACHYKININS IN CNS
AND PERIPHERAL TISSUES.

<u>CNS SITE/PERIPHERAL TISSUE</u>	<u>TACHYKININS pmol g⁻¹</u>		
	SP	NKA	NKB
Cerebral cortex	7.0	4.0	2.9
Striatum	312.2	26.2	1.2
Globus pallidus	114.7	68.9	8.3
Substantia nigra	1154.2	115.0	2.8
Ventral tegmental area	115.4	68.9	17.2
Nucleus accumbens	122.9	70.4	-
Hippocampus	4.1	3.0	1.1
Hypothalamus (lateral nucleus)	201.4	171.3	31.4
Habenula	406.0	240.2	-
Interpeduncular nucleus	474.6	168.3	-
Cerebellum	4.7	1.6	1.6
Solitary nucleus	459.0	389.2	21.7
Raphe nuclei	275.0	83.0	-
Medulla oblongata	226.4	71.1	7.2
Spinal cord			
Dorsal horn (substantia gelatinosa)	503.7	65.9	9.1
Intermediolateral nucleus	121.6	126.0	31.6
Ventral horn	117.4	16.1	2.0
Ileum	47.8	23.9	0.44
Colon	20.1	16.1	0.67
Parotid salivary gland	16.2	0.4	0.4
Submandibular salivary gland	12.5	5.0	0.5
Heart	0.4	0.2	0.3
Urinary bladder	1.7	3.6	0.3
Adrenal gland	1.2	0.8	1.0
Skin	2.5	-	-

TABLE 2. Distribution of tachykinin receptors in selected areas of the central nervous system and selected peripheral tissues (the same locations as used in table 1). As emphasised at the foot of the table, these studies employed two methods, autoradiographic mapping using radiolabelled ligands and hybridisation histochemistry studies mapping the expression of messenger RNA encoding the various receptors. The two techniques have, as described, produced differing results. Data is derived from references cited in text.

TABLE 2.

DISTRIBUTION OF TACHYKININ BINDING SITES
IN CNS AND PERIPHERAL TISSUES.

<u>CNS SITE/PERIPHERAL TISSUE</u>	<u>RECEPTOR BINDING/mRNA</u>		
	NK1	NK2	NK3
Cerebral cortex	0	0*	++++
Striatum	++++	0	0
Globus pallidus	-	-	-
Substantia nigra	0	0	0
Ventral tegmental area	0	0*	+
Nucleus accumbens	+++	0	0
Hippocampus	++	0*	+
Hypothalamus (lateral nucleus)	+	0	0
Habenula	++	0*	+
Interpeduncular nucleus	++	0*	++
Cerebellum	++	0	0
Solitary nucleus	+++	0*	++++
Raphe nuclei	+	0	0
Medulla oblongata	++	0	0
Spinal cord			
Dorsal horn (substantia gelatinosa)	++	0*	++++
Intermediolateral nucleus	++++	0	0
Ventral horn	++	0	0
Ileum	+++	++	+
Colon	++++	++++	+
Parotid salivary gland	+++	0	0
Submandibular salivary gland	++++	0	0
Heart	0	0	0
Urinary bladder	++++	++++	+
Adrenal gland	0	++++	0
Skin	+	0	0

KEY +, ++, +++, ++++ ranked positive identification of binding sites or receptor mRNA.

 0 negative result, ie. receptors not present.

 - no available data.

 0* radiolabelled NKA binding demonstrated at these sites, but NK2 receptor mRNA not detected.

although presumably altering the affinity of the peptide for the different receptor subtypes (Couture et al, 1979, Erspamer, 1981, Regoli et al, 1989).

Prior to the discovery and sequencing of NKA and NKB in the mid 1980's, only two tachykinin receptor subtypes had been proposed : SP-E (for eledoisin) and SP-P (for physalaemin), based on the rank order of potency in various smooth muscle preparations of SP and other structurally related non-mammalian tachykinins. When NKA and NKB were isolated in a pure form it was found that SP was the preferred ligand for the SP-P receptor, NKA for the SP-E receptor and NKB for a previously unidentified receptor subtype, SP-N, at that stage identified in guinea-pig ileum (Laufer et al, 1985, Lee, C.-M. et al, 1986). One study worthy of note, in part because the technique for receptor differentiation is rather unusual is that of Vaught et al (1986). Phenoxybenzamine, at varying doses was used as a multi-affinity receptor antagonist to produce a competitive block of kassinin-, physalaemin-, SP-, NKA-, and NKB-induced contractions of guinea-pig ileum in vitro. From the pA₂ values, three tachykinin receptor subtypes were identified. The same tachykinins were also injected intrathecally in mice, producing a characteristic hind-limb scratching reflex (see later section), and phenoxybenzamine was again employed to block the reflex. In the CNS two distinct tachykinin receptor subtypes were quite clearly revealed, one of which showed selectivity for NKB. The pA₂ values for blockade of NKB effects in the CNS were, however, very different from those seen in the guinea-pig ileum. It is tempting to over-extrapolate the results of the above study. Quite clearly it indicates tachykinin receptor multiplicity in both peripheral and CNS tissues, but it also seems to

indicate tissue or species differences in the NKB or NK3 receptor. This latter conclusion should not be drawn from the data presented, principally from the fact that the peripheral and CNS preparations are very different in themselves, leading to variation in the accessibility of receptor sites to the drugs tested. The variation in experimental conditions relating to the different bioassay preparations used in early studies on tachykinin receptor multiplicity did indeed result in a great deal of confusion. The results of more recent studies, such as that of Guard et al (1990) measuring the binding of radiolabelled and highly selective NK3 ligands, suggest that NK3 receptors in ileum and CNS are pharmacologically identical.

It is now accepted that three tachykinin receptor subtypes exist, almost certainly in both the periphery and the CNS, and no distinction is made between the receptor subtypes for the two areas. NK₁, NK₂ and NK₃ (for neurokinin) are now the most widely accepted terms for the three subtypes, with the receptors showing the highest affinity for SP, NKA and NKB respectively. Neuropeptide gamma has recently been reported as binding preferentially to the NK₂ receptor (Dam et al, 1990b). The ranked affinities of endogenous tachykinins for the three receptor subtypes is (from Helke et al, 1990):

NK ₁	SP>NP gamma>NKA=NPK>NKB
NK ₂	NPK=NP gamma>NKA>NKB>SP
NK ₃	NKB>NKA>SP

Much work has been undertaken to develop specific (as opposed to selective) ligands, both agonists and antagonists, for the receptors. Many of these are analogues of the tachykinins themselves, for example [Sar⁹,Met(O₂)¹¹]-SP (septide), [Nle¹⁰]-NKA (4-10) and [MePhe⁷]-NKB, selective agonists for NK₁, NK₂ and NK₃ receptors respectively. Success has predominantly been achieved in developing receptor agonists with high selectivity (Drapeau et al, 1987a,b, Laufer et al, 1986, Regoli et al, 1987,1988, Rovero et al, 1989, Watson et al, 1983, Wormser et al, 1986). Of the antagonist compounds available, [D-Arg¹,D-Trp^{7,9},Leu¹¹]-SP (spantide), which was one of the earlier antagonists to be developed (Featherstone et al, 1986, Mizrahi et al, 1983), has been the most widely used despite being only partially selective for the NK₁ receptor. Recent drug development has centred on the development of receptor antagonists with better receptor selectivity, many of which are of a non-peptide nature (Advenier et al, 1992, McElroy et al, 1992, Morimoto et al, 1992, Nagahisa et al , 1992, Poncelet et al, 1993, Rovero et al, 1990, Sakurada et al, 1992).

Studies on tachykinin receptor distribution have primarily relied on the binding of [³H] or [¹²⁵I] labelled forms of tachykinins, tachykinin fragments or tachykinin analogues. Early autoradiographic studies confirmed the heterogeneity of tachykinin binding sites in CNS and peripheral tissues (Beaujouan et al, 1986, Burcher et al, 1984, Mantyh et al, 1984b) and established that, in the spinal cord, receptors binding SP were located post-synaptically to sensory terminals in the dorsal horn and also at other sites such as on somatic motor neurones in the ventral horn and on sympathetic pre-ganglionic fibres in the intermediolateral cell column. Later,

more refined investigations, in some cases using agonists with a high selectivity for tachykinin receptor subtypes to displace bound radiolabelled ligand, allowed a more detailed 'map' of receptor distribution to be determined for brain (Beaujouan et al, 1986, Buck et al, 1986, Cascieri et al, 1985, Dam et al, 1990a, Dam and Quirion, 1986, Danks et al, 1986, Mantyh et al, 1984a,b,1989), spinal cord (Hunter et al, 1987, Yashpal et al, 1990,1991) and peripheral tissues (Bergstrom et al, 1987, Mantyh et al, 1988,1989). Studies on peripheral tissues have dealt almost exclusively with the gastrointestinal tract.

The amino acid sequences of the individual tachykinin receptors have also recently been determined, and the distribution of mRNAs encoding the receptors has been mapped in hybridisation histochemistry studies (Hershey and Krause, 1990, Sasai and Nakanishi, 1989, Shigemoto et al, 1990, Tsuchida et al, 1990). The structural characteristics of the three receptor subtypes conferring their differing selectivities for the various tachykinins is now being evaluated, but this work is still at an early stage. It has been suggested that the extracellular domains of the receptors are the most important sequences with regard to binding affinities (Fong et al, 1992a,b,c).

NK₁ receptors are found in both CNS and peripheral tissues, NK₂ receptors predominantly in peripheral tissues and NK₃ receptors mainly in the CNS. Within the brain the density of the different subtypes varies from region to region, with each receptor subtype having a distinct pattern of distribution. In some cases there is a marked mismatch between the presence of tachykinin receptor subtypes and their putative endogenous tachykinin ligand. For example the

substantia nigra contains a large number of irSP containing nerve terminals, as described earlier, but very few NK₁ receptors (Buck et al, 1986, Mantyh et al, 1989). The significance of such mismatches is unclear.

In the spinal cord, the density of NK₁ receptors is highest in laminae I, II and X of the grey matter, and in the intermediolateral cell column, with a much lower presence in the ventral horn. This corresponds closely with the spinal cord distribution of SP. NK₂ receptors (when identified: see below and notes with **table 2**) and NK₃ receptors in the spinal cord are restricted to the dorsal grey matter, and are most likely associated with afferent pathways. In the CNS as a whole, the densities of NK₂ and NK₃ receptors are generally lower than that of the NK₁ subtype. Peripheral tissues generally express both NK₁ and NK₂ receptors, with some tissues (eg. rat myenteric plexus or portal vein smooth muscle) also showing the presence of NK₃ receptors. NK₂ receptors are present in much higher numbers in peripheral tissues than in the CNS; the converse applies to NK₃ receptors.

Some recent studies have failed to detect NK₂ (and in a few cases NK₃) receptors or mRNA in vertebrate CNS (Beaujouan et al, 1991, Dietl et al, 1991) which disagrees with earlier autoradiographic studies where multiple tachykinin receptors were identified in many areas of the CNS. This has led to the hypothesis that the localisation of low numbers of NK₂ receptors in brain and spinal cord was due to poor specificity of the radioligands used in these early studies and that NK₂ receptors may indeed be absent from the CNS. The same argument could be applied to the low levels of NK₃ receptors seen in peripheral tissues. This hypothesis is not

supported by some studies on receptor function at these sites (see later). Tachykinin receptor distribution is summarised in **table 2**.

Tachykinin receptor distribution is clearly complex and the functional roles of the different receptor subtypes have not yet been well established. One note of caution concerns the assumption that each receptor is closely associated with one endogenous tachykinin ligand. NK₁, NK₂ and NK₃ receptors are sometimes referred to as SP, NKA and NKB receptors respectively, although all three tachykinins show some affinity for each receptor subtype. The receptor/ligand mismatches evident in some areas of the brain such as the substantia nigra, where SP is abundant despite the lack of NK₁ receptors, and the observation that NPK and NP gamma both have a higher affinity for the NK₂ receptor than NKA itself, suggest that this association may not be physiologically accurate in many cases.

1.6 TACHYKININ FUNCTIONS AT A CELLULAR LEVEL.

Tachykinin effects at a cellular level can be broadly divided into two categories; 1) elevation of intracellular Ca⁺⁺ concentration and 2) membrane depolarisation, both of which are closely linked to smooth muscle contraction, secretory mechanisms and neurotransmission.

The three receptor subtypes are structurally similar and all show amino acid sequences similar to those found in G-protein coupled receptors such as β -adrenergic or muscarinic receptors. There is much evidence to support the coupling of tachykinin receptors to inositol phospholipid hydrolysis by these G-proteins (Beaujouan et al, 1990, Bristow et al, 1987, Guard et al, 1987, Hanley et al, 1980, Watson,

1984). This would indicate that activation of tachykinin receptors brings about an elevation in intracellular Ca^{++} levels, either by inositol 1,4,5-triphosphate mediated release of Ca^{++} from intracellular stores, or diacylglycerol activation of protein kinase C and the resultant opening of voltage gated Ca^{++} channels (Mayer et al, 1990, Murase et al, 1989).

The principal mechanism of tachykinin induced membrane depolarisation seems to be a reduction in K^+ conductance (Fujisawa and Ito, 1982, Katayama et al, 1979, Nowak and MacDonald, 1982). A variety of membrane effects of tachykinins have, however, been reported. These include inhibition of muscarine-sensitive K^+ current (Adams et al, 1983), inhibition of Ca^{++} -activated K^+ current (Akasu and Tokimasa, 1989) and activation of other, non- K^+ , cation currents (Minota et al, 1981, Reiser et al, 1982). The increases in intracellular Ca^{++} levels outlined above are also reported to enhance Ca^{++} -activated K^+ conductance (Mayer et al, 1990, Murase et al, 1989), which may have some function in balancing the other, depolarising, membrane actions of tachykinins.

1.7 TACHYKININ FUNCTIONS IN PERIPHERAL TISSUES.

As discussed in the earlier section on tachykinin distribution, many peripheral tissues contain nerve fibres or terminals showing immunoreactivity for tachykinins. Such nerve fibres may be of a sensory nature passing directly or indirectly from the peripheral tissue to the CNS, whilst others are intrinsic to the tissue itself or of extrinsic origin, fulfilling an efferent function. Some of these nerve fibres form synaptic contacts within nerve plexi, whilst others contact smooth muscle, secretory tissue, blood vessels or less frequently exist as apparently free nerve endings. The range of tissues in which tachykinins play a functional role is too large to be considered in detail within the scope of this thesis, but the major functions mediated by these peptides in peripheral structures will be outlined with examples of locations at which they operate.

1) Sensory transmission. Tachykinin containing nerve fibres of primary afferent origin can be found in many tissues; eg. skin (Hokfelt et al, 1975a), peri-articular tissues (Pereira da Silva and Carmo-Fonseca, 1990), dental pulp (Olgart et al, 1977), gastrointestinal tract (Domoto et al, 1983, Hayashi et al, 1982), and respiratory tract (Lundberg et al, 1984, Stjarne et al, 1989) and pelvic structures (de Groat, 1987). These fibres are predominantly of the A-delta or C-type, and in many cases have been shown to contain both tachykinins and CGRP, or to be capsaicin sensitive substantiating their classification as primary afferent fibres. These fibres are associated with the transmission of noxious inputs such as chemical or mechanical stimulation, to the CNS.

2) Inflammation. Tachykinins are contained in nerve terminals in the walls of blood vessels where their release brings about plasma extravasation and vasodilation (Markle et al, 1990). Dilation of blood vessels by tachykinins may be indirect and involve the formation of nitric oxide (Whittle et al, 1989). Tachykinin release, in response to noxious stimulation, from the peripheral terminals of primary afferent fibres or in some cases from free nerve endings also contributes to this response, which has been demonstrated in the skin (Yonehara et al, 1987), joints (Lam and Ferrell, 1991,1993) eye (Bill, 1991), dental pulp (Olgart et al, 1977) and lung (Saria et al, 1988). Tachykinins also stimulate the release of histamine from mast cells and act as trophic factors for leucocyte infiltration (Coulson and Holden, 1990) and further sensitise peripheral tissues to the inflammatory effects of other compounds such as prostaglandin E₂ and prostacyclin (Nakamura-Craig and Smith, 1989). This sensitisation results in further build up of inflammatory fluid, promotes more release of tachykinins from peripheral nerve endings and also lowers the threshold of these peripheral nociceptors, resulting in the pain which is frequently associated with inflammatory change. Tachykinin containing fibres may also be closely associated with antibody production in response to antigenic challenge in the immune system (Eglezos et al, 1991)

3) Secretion. Tachykinins have been shown to both stimulate and inhibit secretion. Indeed SP was first identified by its sialogogic nature. In salivary glands in the rat and ferret, SP has been shown to be involved with endogenous stimulation of salivary glands.

Overall control of salivary output also seems to involve acetylcholine, however, as atropine is required in addition to tachykinin antagonists to completely abolish the outflow of saliva produced by nerve stimulation in both the sub-mandibular and parotid glands (Ekstrom et al, 1988,1989, Hanley et al, 1980). In the rat pancreas SP inhibits fluid secretion from duct cells (Ashton et al, 1990) but stimulates amylase secretion from acinar cells (Kato et al, 1984). SP also stimulates mucus secretion in the respiratory tract (Coles et al, 1984).

4) Cardiovascular system. Vasodilatation and plasma extravasation produced by tachykinins have been discussed above. It has been suggested that at some sites such as heart (Weihe et al, 1981), skeletal muscle (Ohlen et al, 1987) and in the gastro-intestinal tract (Donnerer et al, 1984), SP may be involved in autoregulation of blood flow. Changes in mean arterial blood pressure, heart rate and peripheral vascular permeability have all been seen following intrathecal or systemic administration of tachykinins (Eimerl et al, 1985, Hassessian et al, 1987,1988). These effects are most likely mediated via spinal NK₁ receptors located on parasympathetic preganglionic fibres although the effector mechanisms were not determined in these studies.

5) Smooth muscle. In the respiratory system, tachykinins are potent constrictors of smooth muscle in the major airways, where NKA seems to be the most important of the endogenous tachykinins (Saria et al, 1988). The gastrointestinal tract, in addition to large numbers of sensory fibres or fibres of extrinsic origin showing

immunoreactivity for tachykinins (Costa et al, 1980, Hayashi et al, 1982, Lundberg et al, 1978), also contains the peptides in many intrinsic fibres of the myenteric and submucous plexi (Franco et al, 1979, Malmfors et al, 1981, Nilsson et al, 1975a, Pearse and Polak, 1975). Exogenously applied tachykinins clearly have an effect on motility patterns in the smooth muscle of the gut wall (Kawahara and Yanaihara, 1987) and populations of tachykinin containing fibres may play an important part in regulating gut motility, although both NK_B and NK₃ receptors may be of lesser significance at this site than the other tachykinins and receptor subtypes. Tachykinin functions in these and other peripheral tissues are summarised in **table 3**.

TABLE 3. Some postulated roles for tachykinins in peripheral tissues. The (ubiquitous) association of tachykinins with primary afferent pathways has been separated. Although not specified in the table, most studies have been on the functions of substance P.

TABLE 3.
SOME POSTULATED ROLES FOR TACHYKININS
IN PERIPHERAL TISSUES.

GENERAL	neurotransmitters of afferent pathways (often associated with small diameter fibres conveying ?nociceptive input.)
EYE	afferent pathways from cornea, miosis, retinal function.
TEETH	vasodilation in dental pulp.
SALIVARY GLANDS	increase secretion.
GASTRO-INTESTINAL TRACT	control of smooth muscle contraction, local regulation of blood flow, neurotransmitters of enteric nervous system , antiulcerogenic.
GALL BLADDER	control gall bladder contraction/rate of bile secretion by hepatocytes.
PANCREAS	stimulate amylase secretion in acini, inhibit fluid secretion in duct cells.
RESPIRATORY SYSTEM	cause bronchoconstriction, mucus secretion, increased mucociliary response, vasodilation.
CARDIOVASCULAR SYSTEM	involved in regulation of coronary blood flow, vasodilation, plasma extravasation, hypoxic response of carotid body.
URINARY SYSTEM	associated with vasodilation, diuresis, natriuresis, contraction of urinary bladder smooth muscle, afferent pathways triggering micturition reflex.
ADRENAL GLANDS	<i>modulation.</i> modulate of catecholamine release.
SKELETAL MUSCLE	involved in regulation of local blood flow.
BONES and JOINTS	important mediators of inflammation in periarticular structures.
SKIN	associated with control of blood flow, sweat glands.
INFLAMMATORY RESPONSES	vasodilation, plasma extravasation, flare and wheal (triple response), activation of white blood cells.
IMMUNE SYSTEM	proliferation of lymphocytes, increase in immunoglobulin A and M synthesis, increase in lymph flow from peripheral lymph nodes.
HEALING	? proliferation of endothelial cells, ? increased rate of DNA synthesis.

1.8 TACHYKININ FUNCTIONS IN THE CENTRAL NERVOUS SYSTEM.

I.SITES OUTWITH THE SPINAL CORD/AUTONOMIC NERVOUS SYSTEM.

As with tachykinin functions in peripheral tissues, the roles of tachykinins in CNS sites outwith the spinal cord are too complex to be discussed at length within the scope of this thesis, although they have recently been reviewed by Otsuka and Yoshioka (1993). Key observations will, however, be made.

1)Control of dopamine release. Both SP and NKA increase the release of dopamine from neurones arising in the substantia nigra and ventral tegmental area (A10). (Kelley et al, 1979, Reid et al, 1988). The dopaminergic neurones from these sites target many areas of the CNS including the striatum and are closely associated, in man at least, with motor control. Nigral SP levels have been shown to be reduced in cases of Parkinson's disease in man (Mauborgne et al, 1983). It has already been noted that despite the high levels of irSP present in the substantia nigra (Brownstein et al, 1976, Cooper et al, 1981, Douglas et al, 1982, Emson et al, 1980, Gale et al, 1978, Kanazawa and Jessell, 1976, Lovick and Hunt, 1983), there are few tachykinin binding sites in this area (Buck et al, 1986, Mantyh et al, 1989). This mismatch cannot currently be explained. In the nucleus accumbens, SP and NKA have both been shown to enhance the actions of endogenous dopaminergic neurones (Elliot et al, 1986, Van den Bos et al, 1990). It is again worth noting that dopaminergic systems have been implicated in the modulation of the rate of PPTA

gene transcription (Bannon et al, 1987, Lindefors et al, 1989), suggesting some form of reciprocal control between the two systems.

2)Noradrenergic neurones of the locus coeruleus. Nerve terminals containing SPLI make synaptic contact with noradrenaline-containing neurones of the locus coeruleus, one of the major sites of origin of catecholaminergic neurones in the brain, (Pickel et al, 1979). These neurones, many of which pass into the spinal cord as descending inhibitory fibres (Westlund et al, 1981), are excited by direct application of SP (Guyenet and Aghajanian, 1977).

3)Centres of respiratory and cardiovascular control. Like the substantia nigra, the nuclei of the solitary tract contain high levels of tachykinins, especially SP (see references in paragraph 1 above). This area of the brain controls the transmission of many respiratory and cardiovascular reflexes. Tachykinin containing terminals in the solitary nuclei constitute fibres arising from other areas of the brain, such as the raphe nuclei and ventral medulla (Thor and Helke, 1987) and also represent primary afferent fibres running in cranial nerves V, IX and X (Helke et al, 1980). Some of these afferent fibres may arise from chemoreceptors or baroreceptors in, for example, the carotid sinus. Microinjection of SP into this area causes a brief drop in blood pressure and slowing of the heart rate (Hall et al, 1989) and also increases respiratory tidal volume and frequency (Chen et al, 1990).

4)Autonomic nervous system. Neural elements showing tachykinin immunoreactivity can be found in the dorsal nucleus of the vagus in the brainstem, and also in the intermediolateral and sacral parasympathetic nuclei in the spinal cord (Cuello and Kanazawa, 1978, Hancock and Peveto, 1979). These are all parasympathetic preganglionic nuclei. Application of SP to individual parasympathetic preganglionic fibres tends to cause their depolarisation (Dun and Mo, 1988). The functions of tachykinins in this system may be illustrated by the changes in mean arterial blood pressure, heart rate and peripheral vascular permeability which have been seen following intrathecal administration of tachykinins (Hassessian et al, 1987,1988).

1.9 TACHYKININ FUNCTIONS IN THE CENTRAL NERVOUS SYSTEM.

II. SPINAL CORD NOCICEPTIVE PROCESSING.

The evidence to support a role of tachykinins in spinal processing of nociceptive inputs is overwhelming. SP, NKA and NKB all elicit a characteristic grooming or scratching reflex when administered intrathecally in rodents (Gamse and Saria, 1986, Wilcox, 1988), thought to relate to the perception of pain. Intrathecal administration of SP or NKA also decreases the latency of the rat tail-flick reflex (Cridland and Henry, 1986), whereas tachykinin depletion by capsaicin pretreatment in the same species brings about a thermal analgesia (Yaksh et al, 1979). Capsaicin is thought to have a selective effect on primary afferent structures. Capsaicin

application initially stimulates the release of, and subsequently depletes, not only tachykinins but other peptides which exist in primary afferent structures, such as CGRP and somatostatin (Hua et al, 1986, Saria et al, 1986, Yaksh et al, 1979). As only approximately 20% of dorsal root ganglion neurones show tachykinin immunoreactivity (Hokfelt et al, 1976), the behavioural effects of capsaicin may clearly relate to its effects on other systems apart from those involving tachykinins.

If the tachykinins function as neurotransmitters in pain pathways then they should fulfil the basic criteria for neurotransmitters in general; both the peptides and their receptors should be present at appropriate sites, their release should be demonstrated following appropriate stimuli, some effect on post synaptic structures should be evident and some mechanism to limit their action should exist.

1) Peptide/receptor distribution. These topics have been discussed in earlier subsections. It might again be noted that tachykinins have been identified in synaptic vesicles in nerve terminals within the spinal cord (Cuello, 1977, Cuello and Kanazawa, 1978, Merighi et al, 1989, Pelletier et al, 1977), and that tachykinin immunoreactivity in the spinal cord is largely associated with fibres of primary afferent origin. In the spinal cord, unlike some areas of the brain such as the substantia nigra, there is a good correlation between tachykinin peptide distribution and tachykinin receptor distribution.

2) Tachykinin release. Superfusion studies have been used to detect release of SPLI from spinal cord preparations in the rat, guinea pig and cat, both in vivo and in vitro (Hua et al, 1986, Linderoth and Brodin, 1989, Ono et al, 1991, Otsuka and Konishi, 1976, Pang and Vasko, 1986, Saria et al, 1986, Theriault et al, 1979, Yaksh et al, 1979, 1980). These studies initially demonstrated raised levels of SPLI in the superfusate only in response to capsaicin or veratridine application and to high levels of K^+ . Release of SPLI in response to high K^+ levels was Ca^{++} dependant, being abolished by low Ca^{++} /high Mg^{++} , (such Ca^{++} dependant release of tachykinins can also been demonstrated in various brain regions in vitro, Lindefors et al, 1985). Electrical stimulation of dorsal roots or peripheral nerves at A-delta or C-fibre strength was inconsistent in producing raised levels of SPLI (stimulation of larger fibres was ineffective) and peripheral noxious stimuli, whether mechanical, thermal or chemical produced no detectable changes.

The findings of such studies must be interpreted with some caution on two counts. Firstly the sensitivity of simple tissue perfusion techniques may be too low to detect small increases in tachykinin levels following 'natural' peripheral stimuli such as pinching or heating of the skin. Thus only relatively harsh stimuli, such as capsaicin application or electrical stimulation, affecting large populations of neurones produce detectable changes in levels of released tachykinins. It would then be premature to conclude that discrete peripheral stimuli in vivo are physiologically ineffective in eliciting tachykinin release in the spinal cord. Secondly, elevation of extracellular K^+ levels will stimulate all neurones in spinal cord slice or similar preparations. It has already been

pointed out, in the section dealing with tachykinin distribution, that approximately 50% of nerve terminals showing tachykinin immunoreactivity in the spinal cord may be of intrinsic or supra-spinal origin. Release of tachykinins in the spinal cord following elevation of K^+ or similar 'blanket' stimuli cannot then be attributed solely to release from primary afferent terminals.

Modifications of the superfusion technique, namely the push-pull cannula in the rabbit and rat (Kuraishi et al, 1983, 1985a, Oku et al, 1987, Tiseo et al, 1990) and microdialysis in the cat (Brodin et al, 1987), have been used to detect raised levels of tachykinin immunoreactivity in the spinal cord in response to peripheral noxious mechanical and thermal stimuli, inflammation of peripheral tissues and to peripheral C-fibre stimulation. These techniques have a better sensitivity for peptide detection than simple superfusion, presumably accounting for their success in detecting elevated levels of tachykinin release in response to physiological noxious peripheral stimuli. Their anatomical resolution is not, however, sufficient to allow detailed evaluation of sites of release in vivo (resolution is in the order of 200 μ m). Evoked tachykinin release in these studies could still be derived from intrinsic neurones of the spinal cord rather than from primary afferent terminals.

In recent years a technique for peptide detection in vivo, the **antibody microprobe technique**, has been developed by Duggan and co-workers (Duggan et al, 1988a, Duggan and Hendry, 1988, Hendry et al, 1988). This was the technique used in the experiments detailed in this thesis and the methodology will be discussed at length in a later subsection. It is, however, worth noting at this point that antibody microprobes have very high spatial resolution (<100 μ m) and

sensitivity for peptide detection (in the order of 10^{-17} moles peptide per microprobe). In a variety of studies antibody microprobes have demonstrated release of SP and NKA in cat spinal cord in response to a variety of peripheral noxious stimuli; thermal, mechanical, and chemical (arthritic) stimulation of cutaneous or articular structures and electrical stimulation of afferent fibres of nociceptive origin. (Duggan et al, 1988b, 1989, 1990, 1991, 1992, Hope et al, 1990b, c, Lang et al, 1991, 1994, Morton et al, 1990, 1991, Schaible et al, 1990, 1992). Non-noxious peripheral stimuli were ineffective in evoking spinal release of tachykinins. By merit of their high spatial resolution, antibody microprobes further demonstrated that noxious stimulus-evoked release of tachykinins is centred on the substantia gelatinosa, laminae I and II of the superficial dorsal horn, the major site of primary afferent fibre termination in the spinal cord. This supports the hypothesis that tachykinins are released from the central terminals of fibres of nociceptive origin.

3) Effects of tachykinins on spinal cord structures. The cellular mechanisms underlying the effects of tachykinins on neuronal structures have already been discussed.

The earliest observations on SP function in spinal cord was simply that it excited neurones in spinal cord in vivo (Tulloch and Zieglgansberger, 1978) which in some cases were shown to be activated by peripheral noxious stimuli (Randic and Miletic, 1976, Sastry, 1979b). SP applied to cultured spinal neurones produces a slow depolarisation, lasting many seconds (Nowak and MacDonald, 1982). Similar long lasting excitatory post-synaptic potentials (EPSPs) are seen in neurones of the cat spinal cord neurones following cutaneous



noxious stimulation, and such EPSPs can be blocked by systemic administration of tachykinin antagonists (De Koninck and Henry, 1991). In an isolated spinal cord/tail/hindlimb preparation from newborn rat, the tachykinin antagonist spantide blocks similar long lasting depolarisations recorded in neurones of the ventral horn in response to electrical C-fibre stimulation in the dorsal horn or to peripheral capsaicin application (Nussbaumer et al, 1989, Otsuka and Yanagisawa, 1988). NKA application produces similar electrophysiological changes in spinal neurones to those seen with SP (Yanagisawa and Otsuka, 1990).

It is interesting to note that a proportion of tachykinin containing primary afferent terminals in the spinal cord also contain the fast acting excitatory neurotransmitter, glutamate (Battaglia and Rustioni, 1988, de Biasi and Rustioni, 1988). Stimulation of such terminals should then result in both slow and fast post-synaptic potentials, the significance of which will be discussed at the end of this subsection. Within the spinal cord tachykinins have, then, been shown to function as excitatory transmitters in primary afferent pathways of pain transmission (in addition to their roles in other systems such as autonomic preganglionic pathways). Is the role of tachykinins in primary afferent pathways limited to fibres of nociceptive origin?

Henry (1976) initially reported that SP only excited dorsal horn neurones which responded to noxious cutaneous thermal stimuli, and more recently Duggan et al (1988b,1990) have shown that noxious mechanical, thermal and chemical stimuli are all effective in evoking spinal release of both SP and NKA, whereas innocuous stimuli do not evoke such release. Such a nocispecific role for tachykinins in

primary afferent transmission is supported by reports that dorsal horn neurones with strong nociceptive input show significantly higher numbers of tachykinin immunoreactive contacts in the spinal cord than other neurone types (De Koninck et al, 1992). The hypothesis is, however, not supported by the findings of Leah et al (1985), who reported no correlation between sensory function of electrophysiologically characterised primary afferent fibres and SP-immunoreactivity. Many fibres in this study did not respond to any form of cutaneous stimulation; these may have been **silent nociceptor** fibres, which under normal conditions do not respond to defined peripheral stimuli but only become sensitive to noxious stimulation under inflammatory conditions (Handwerker et al, 1991). Duggan and co-workers have shown that spinal release of SP and NKA in response to peripheral noxious stimuli is enhanced during inflammation (Hope et al, 1990b, Schaible et al, 1990). This enhanced release may be derived from such silent nociceptors and may in part explain the findings reported by Leah and co-workers.

As with other systems, the specific roles of the different tachykinins and their receptors in spinal afferent processing have not yet been well established. Two recent studies are, however, worth noting. An antinociceptive effect of intrathecally applied NKB on the rat tail-flick reflex has been reported (Laneauville et al, 1988) leading to the suggestion that NK₃ receptors might mediate antinociceptive functions in the spinal cord. The same group, however, reported a variable effect of SP, both algesic and analgesic, in the same preparation and the observation must therefore be approached with some caution. The lack of an NK₃ mediated component in the tachykinin facilitation of thermal nociception (but

not antinociception per se) in the rat has also been reported (Fleetwood-Walker et al, 1988).

4 Tachykinin metabolism/reuptake mechanisms. Although an uptake mechanism has been demonstrated in CNS for the tachykinin fragment SP (5-11) (Nakata et al, 1981), a similar mechanism does not appear to exist for the whole SP (Segawa et al, 1977). Indeed no selective reuptake mechanism has been identified for any of the mammalian tachykinins (McKelvey and Blumberg, 1986). Another way in which tachykinin function in vivo could be limited is by enzymatic degradation.

In vitro, SP and NKA are degraded by a number of enzymes including angiotensin converting enzyme (ACE), aminopeptidases, carboxypeptidases and enkephalinase (EC 3.4.24.11) (Littlewood et al, 1988, Turner and Hooper, 1987, Wang et al, 1991). Acetylcholinesterase will also degrade SP in vitro (Chubb et al, 1980) but this effect has not been demonstrated under in vivo conditions. Inhibition of enkephalinase activity has been shown to potentiate the detection of SP release from slices of rat substantia nigra and guinea-pig spinal cord in vitro (Geppetti et al, 1989, Mauborgne et al, 1987a) and to facilitate the effects of exogenously applied SP or NKA in peripheral tissues such as the ferret trachea (Sekizawa et al, 1987).

Several peptidases can be detected in mammalian nervous tissue or cerebrospinal fluid (Barnes et al, 1991, Healy and Orlowski, 1992, Nyberg et al, 1984). Although the exact identity of the enzyme(s) responsible for actual tachykinin degradation in vivo has not been established, Duggan et al (1992) reported that kelatorphan, an

inhibitor of aminopeptidases, dipeptidyl-aminopeptidases and enkephalinase, facilitated the spread of SP in the cat dorsal horn following noxious stimulus-evoked release and that this facilitation was not further increased by the application of captopril, an inhibitor of ACE. It was postulated that the marked spread of NKA seen following its release under normal conditions (Duggan et al, 1990) might relate to its resistance to proteolytic degradation in vitro, which is greater than that seen for SP (Bishop et al, 1986, Theodorsson-Norheim et al, 1985). NKA has a half-life, in vitro, in the order of 30 minutes, whereas that of SP is only about 2 minutes.

The distribution of enkephalinase immunoreactivity in the rat CNS correlates well with the distribution of irSP in many (but not all) areas, including the superficial laminae of the spinal cord (Pollard et al, 1989). Of the potential mechanisms of tachykinin degradation, a role for enkephalinase in vivo is currently the best substantiated.

5) Conclusions. The role of tachykinins as neurotransmitters is well supported by the results of the investigations outlined above. These peptides fulfil all of the necessary criteria for their classification as neurotransmitters; presence, presence of their receptors, evident release and function at both cellular and system levels. These criteria have been demonstrated most comprehensively in the dorsal spinal cord, where their role in primary afferent transmission has been conclusively established. Within spinal afferent pathways both SP and NKA can be linked very selectively to primary afferent pathways of nociceptive origin, although the physiological role of NKB is perhaps not so clear.

The one area, however, where SP and NKA do not conform to the pattern of a classical neurotransmitter is in their durations of action and the probable mechanisms and rates of their breakdown in vivo: this is especially true of NKA. Glutamate, which may be co-released with the tachykinins from a proportion of primary afferent terminals produces 'fast' signalling from one neurone to another and is removed rapidly from the site of its release (Johnston, 1978). NKA, and to a much lesser extent SP, persist beyond the duration of the stimuli evoking their release and spread away from the initial area of that release. In this respect they may represent a second form of neurotransmission, which has been termed "volume transmission" (Fuxe and Agnati, 1991). Released tachykinins may, then, reach receptor sites distant from the initial area of release, and their effects may continue for some time following that release. The ligand/receptor mismatch described in the substantia nigra may be evidence for such a mechanism. Physiologically, NKA acting at tachykinin receptors in the rat spinal cord has been shown to potentiate the flexor withdrawal reflex for more than one hour following its release (Wiesenfeld-Hallin et al, 1991).

One recent study (Schaible et al, 1992) demonstrated that microinjection of CGRP, itself contained in primary afferent terminals of nociceptive origin, directly into the superficial dorsal horn of the cat spinal cord produced similar increases in spread and persistence of released SP as those seen following peptidase inhibition. This lends more support to the hypothesis of volume transmission, as it may indicate that such a mechanism of neurotransmission could also be under some form of physiological control in vivo.

The observations that SP and NKA in the spinal cord may not act focally and rapidly do not detract from their close association with nociceptive primary afferent transmission. It might be, however, that these peptides would be better described as neuromodulators, altering spinal cord function over a comparatively wide area and for comparatively protracted periods, as opposed to classical neurotransmitters such as glutamate.

1.10 ANTIBODY MICROPROBES

I. PRINCIPLES OF THE TECHNIQUE.

Antibody microprobes were developed (Duggan and Hendry, 1988, Duggan et al, 1988a)

"as a means of getting better spatial resolution in determining sites of release of neuropeptides in the central nervous system and to do so with minimal disturbance to the structures giving release" (Duggan, 1992).

The technique has enabled the release of tachykinins (Duggan et al, 1988b, 1989, 1990, 1991a,b, 1992, Hope et al, 1990b,c, Lang et al, 1991, 1994, Lang and Hope, 1994, Morton et al, 1990, Schaible et al, 1990, 1992, 1993), and of other peptides such as CGRP (Morton et al, 1990), somatostatin and nerve growth factor (Duggan et al, 1989, Morton et al, 1988), galanin (Hope et al, 1994), and dynorphin (Riley et al, current study) in response to a variety of peripheral stimuli, to be evaluated in the spinal cord of either the cat or the rat in vivo. The technique has also been used in the rat to measure β -endorphin release in the periaqueductal grey, (Duggan et al, 1993), thyrotrophin releasing hormone in the lateral septum and septo-hypothalamic nucleus (Waterfall et al, 1993) and SP release in the

nucleus accumbens and striatum (Furmidge et al, 1993,1994a,b). In the cat brain, antibody microprobes have been used to measure enkephalin, neuropeptide Y (NPY) and SP release in the periaqueductal grey and (SP only) ventrolateral medulla (Williams et al, 1992,1993,1994).

The preparation and use of antibody microprobes will be discussed fully in the section on experimental methods. Briefly, antibody microprobes are glass microelectrodes, of similar dimensions to those used for extracellular recording in electrophysiological studies, evenly coated with antibodies raised against a particular peptide. Microprobes are inserted, with stepping motor micromanipulators, sequentially into the tissue of interest with the concurrent application of defined peripheral stimuli. Released peptide will then bind to antibodies on the area of the microprobe surface adjacent to the site of release. Such binding of endogenous ligand can then be detected by the subsequent failure of the microprobe to bind an exogenous, radiolabelled form of the peptide. The distribution of the radiolabelled ligand is measured with a computer aided image analysis system as the optical density of autoradiographs obtained by incubating the radiolabelled microprobes on x-ray film. Individual microprobe analyses are grouped together according to stimulus/experimental parameters, and pairs of such grouped or 'mean' image analyses can be compared statistically. The release pattern of endogenous ligand detected on the antibody microprobes can then be related back to the individual laminae of the spinal cord, or other discrete CNS structures. The principles of the antibody microprobe technique are illustrated in **figures 4 - 7**.

FIGURE 4. A. Reaction of the glass surfaces of antibody microprobes with γ -Aminopropyltriethoxysilane to produce alkylamine (siloxane coated) glass microprobes.

B. The sequence of reagents used to couple immunoglobulins to siloxane coated microprobes.

FIGURE 4.
PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE (1) .

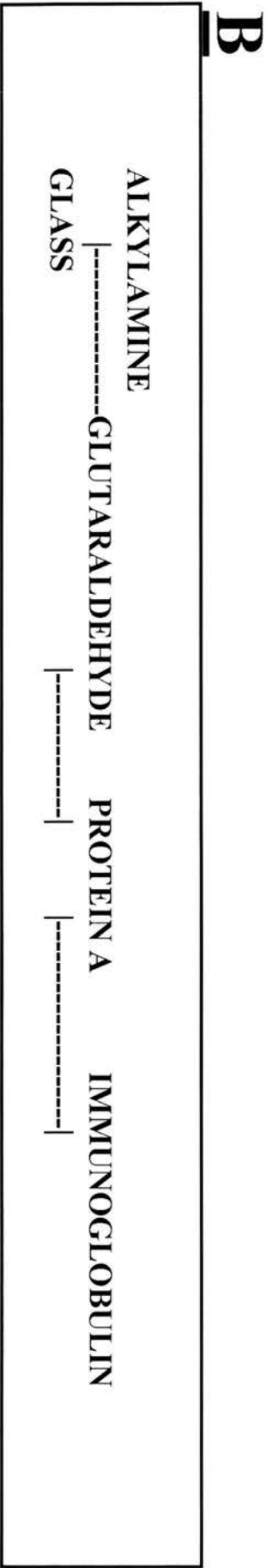
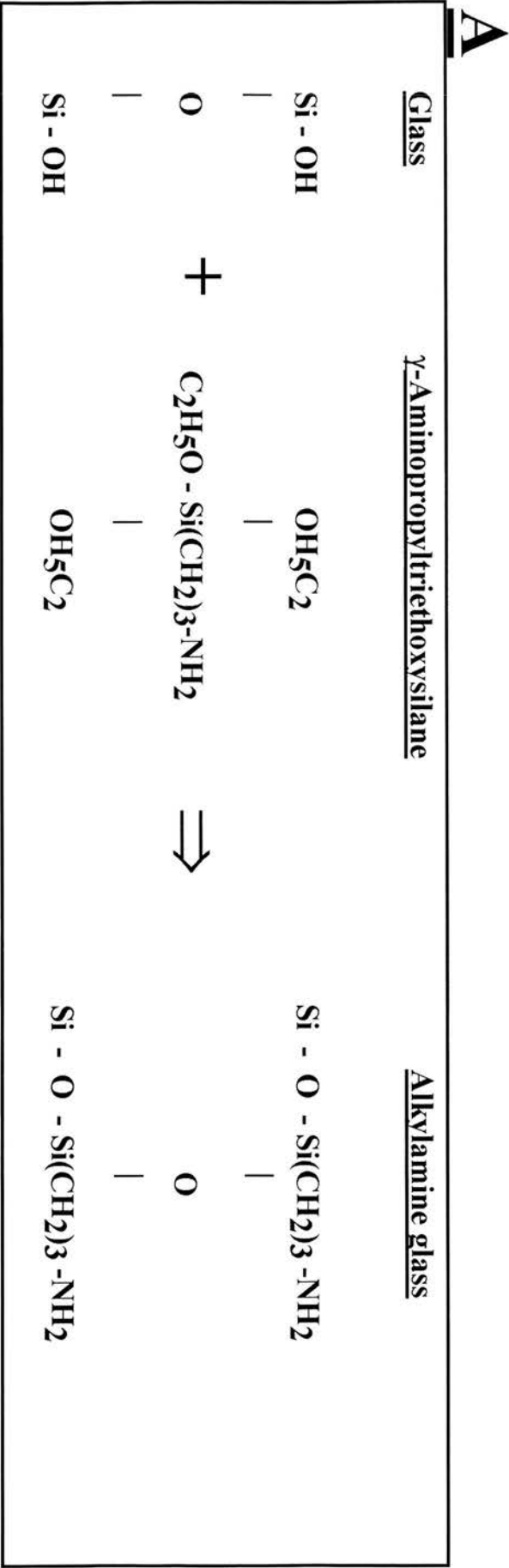


FIGURE 5. Principles of neuropeptide detection in vivo using antibody microprobes, from antibody binding to the production of autoradiographic images.

FIGURE 5.
PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE (2) .

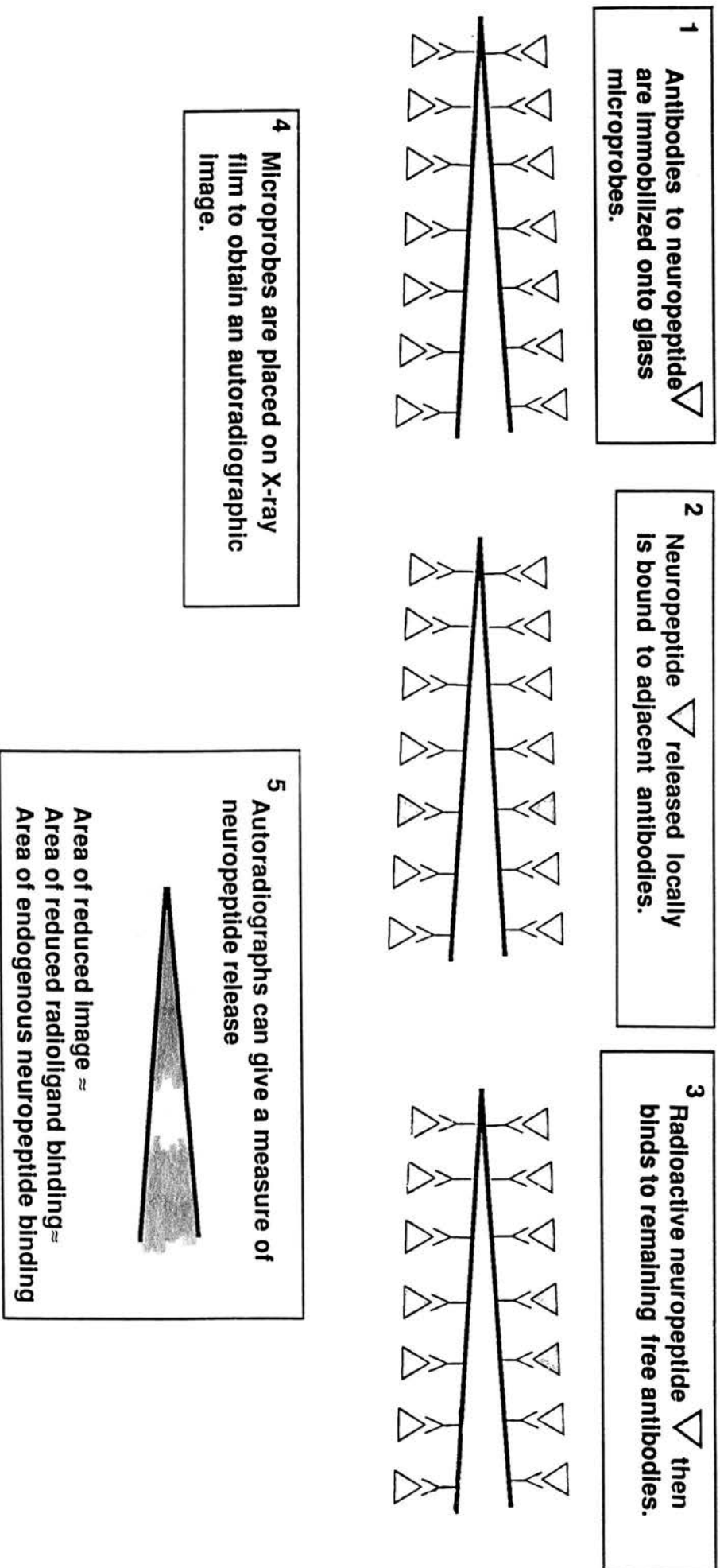


FIGURE 6. Conversion of single autoradiographic images into grey scale plots and their grouping into mean image analyses with means and standard errors of means.

FIGURE 6.
PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE (3).

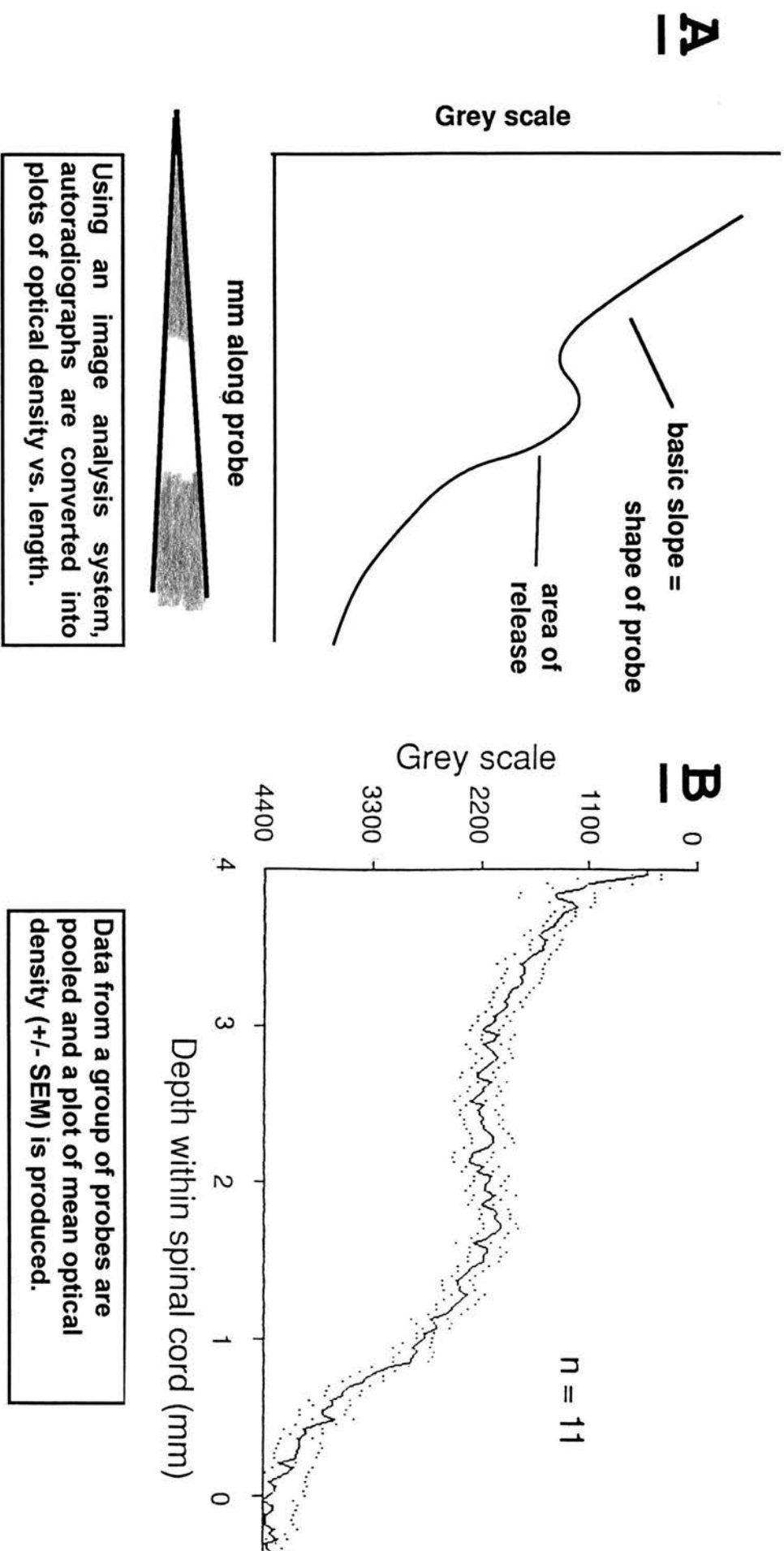
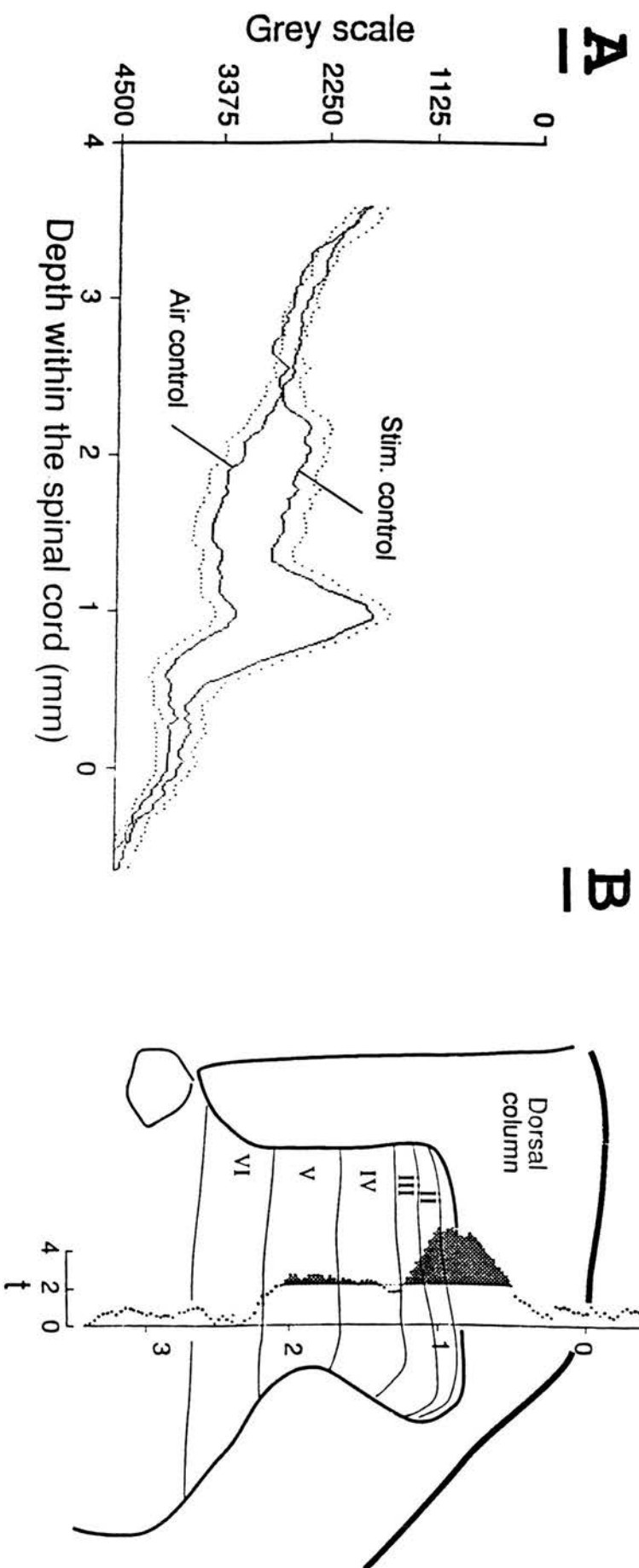


FIGURE 7. Statistical comparison between two mean image analyses and the relation of statistical differences to sites within the spinal cord.

FIGURE 7.
PRINCIPLES OF THE ANTIBODY MICROPROBE TECHNIQUE (4) .



Mean image analyses for two groups of microprobes can be compared statistically, using the Student's t-test. Areas of statistically significant differences in level of neuropeptide detection can be referred to depth within spinal cord.

Although not a truly quantitative technique, increases or decreases in the total amount of peptide released over a period of time can be visualised, as the non-damaging nature of the microprobes means that many individual probes can be placed, one after another, in the same area of tissue. Thus, antibody microprobes have been used to demonstrate the altered release pattern of noxious stimulus-evoked SP release in cat spinal cord, following local administration of peptidase inhibitors or calcitonin gene-related peptide (Duggan et al, 1992, Schaible et al, 1992) or following the development of an acute experimental arthritis (Schaible et al, 1990). The antibody microprobe is currently the most sensitive and least damaging technique available for the in vivo detection of peptide release. Antibody microprobes might be described as a semi-quantitative, semi-dynamic means of evaluating neuropeptide release in vivo, and are ideally suited to the aims of the investigations presented in this thesis, as outlined at the end of this section.

1.11 ANTIBODY MICROPROBES

2) PATTERNS OF TACHYKININ RELEASE

IN CAT SPINAL CORD.

Antibody microprobes have been used to investigate the stimulus-evoked release of both SP and NKA in the dorsal spinal cord of the cat (Duggan et al, 1988b,1989,1990,1991a,b,1992, Hope et al, 1990b,c, Lang et al, 1991,1994, Lang and Hope, 1994, Morton et al, 1990, Schaible et al, 1990,1992,1993). Noxious peripheral thermal, mechanical and electrical (C-fibre strength) stimuli are all effective in evoking the release of both peptides, the normal release

patterns of which are illustrated in **figures 8 and 9**. Innocuous peripheral stimuli are normally ineffective in evoking spinal release of tachykinins although, in a model of acute arthritis, flexion of the arthritic joint to a degree non-noxious in the normal animal did evoke release of both NKA (Hope et al, 1990b) and SP (Schaible et al, 1990). Furthermore, the pattern of irSP detection in the spinal cord in response to flexion of an arthritic joint was different to that seen in the normal animal, but similar to the pattern of SP release seen following intraspinal application of peptidase inhibitors (Duggan et al, 1992) or CGRP (Schaible et al, 1992) (see below and **figure 10**).

The characteristics of stimulus-evoked SP and NKA release in the cat spinal cord are outlined in the following pages.

Stimulation of normal peripheral tissues.

NKA. 1) Release in the spinal cord is evoked by noxious peripheral mechanical, thermal and electrical stimulation.

2) irNKA is detected along the entire length of the antibody microprobe following its stimulus evoked release. This detection extends both ventrally (measured as deep as lamina V) and dorsally (as far as the spinal cord surface) from the substantia gelatinosa (SG) of the spinal dorsal horn, the major site of primary afferent fibre termination. As irNKA is detected in the white matter dorsal to the SG, where there are very few, if any, NKA containing nerve terminals, it has been proposed that this pattern of post stimulus irNKA detection represents an initial focal release in the superficial laminae of the dorsal horn followed by the subsequent diffusion of the peptide away from the initial area of release.

3) Basal levels of irNKA are detected in the spinal cord, at all depths, in the absence of any prior peripheral noxious stimulation.

4) Following its stimulus-evoked release, irNKA persists in the spinal cord, and significantly elevated levels of the peptide can be detected for between 30 minutes and 1 hour following the cessation of the stimulus.

SP. 1) Release in the spinal cord is evoked by noxious thermal, mechanical and electrical stimulation. Early work suggested that relatively harsh noxious stimuli (compared to those employed in irNKA studies) were required to elicit spinal cord release of irSP (Duggan et al, 1988b). This may relate to the sensitivity of the antibody microprobes, or rather to the antisera used, in these early studies. Broadly speaking, the stimulus parameters required to evoke irSP and irNKA release in the spinal cord are the same.

2) Stimulus evoked release of irSP is much more focal than that seen with irNKA. irSP release is primarily focused on the SG, laminae I and II, of the superficial dorsal horn. A smaller area of release is located approximately 0.5mm deep to this in laminae III/V. In some individual animals, at some spinal cord sites, very small (100-200µm) foci of irSP release are detected in the deep dorsal horn (personal observation, unpublished). irSP detection on the surface of the spinal cord is also encountered in some individuals but it is unclear whether this relates to the applied peripheral noxious stimuli or simply to inflammation of the surrounding pial structures (Schaible et al, 1990).

FIGURE 8. Typical pattern of stimulus-evoked immunoreactive substance P release.

A. compares two mean image analyses for groups of microprobes. Microprobes were inserted 3mm into the dorsal spinal cord for 10 minutes during either electrical stimulation of the ipsilateral tibial at C-fibre strength ('Stim. control', n=18) or in the absence of peripheral stimulation ('Air control', n=17). The mean grey scale plot for each group is illustrated, with the + and - standard errors, respectively for the two groups, calculated at 30µm intervals.

B. Plot of the results of a Student's t-test performed between paired data points from the two groups at 30µm intervals. Points at which the difference attained statistical significance ($t > 2$, $P < 0.05$) are shaded in grey and referred to a diagram showing the locations of the different laminae of the dorsal horn. The typical pattern of stimulus-evoked substance P detection is revealed, ie. highly significant levels of substance P in a confined band centred on the substantia gelatinosa, laminae II and III, with a less well defined band in the deeper laminae, IV and V, of the dorsal horn.

FIGURE 8.
NORMAL DISTRIBUTION OF SUBSTANCE P DETECTION.

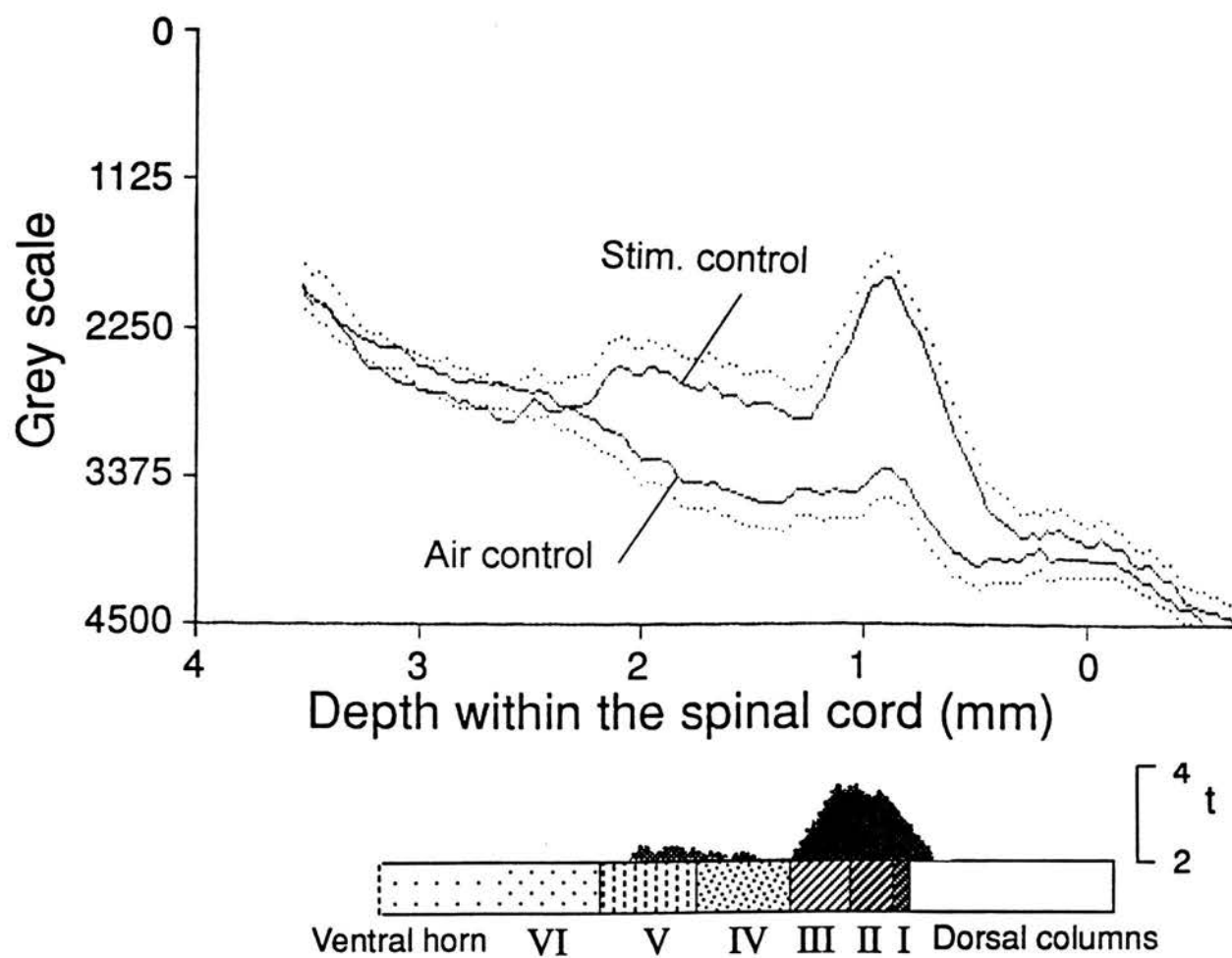


FIGURE 9. Typical pattern of stimulus-evoked immunoreactive neurokinin A release.

A. compares two mean image analyses for groups of microprobes. Microprobes were inserted 3mm into the dorsal spinal cord for 30 minutes during either electrical stimulation of the ipsilateral tibial at C-fibre strength ('Stim. control', n=7) or in the absence of peripheral stimulation ('Air control', n=11). The mean grey scale plot for each group is illustrated with the positive and negative standard errors, for the two groups respectively, calculated at 30 μ m intervals.

B. Plot of the results of a Student's t-test performed between paired data points from the two groups at 30 μ m intervals. Points at which the difference attained statistical significance ($t > 2$, $P < 0.05$) are shaded in grey and referred to a diagram showing the locations of the different laminae of the dorsal horn. The typical pattern of stimulus-evoked neurokinin A detection is revealed, ie. highly significant levels of neurokinin A throughout the entire dorsal horn of the grey matter and also extending dorsally into the white matter.

FIGURE 9.
NORMAL DISTRIBUTION OF NEUROKININ A DETECTION.

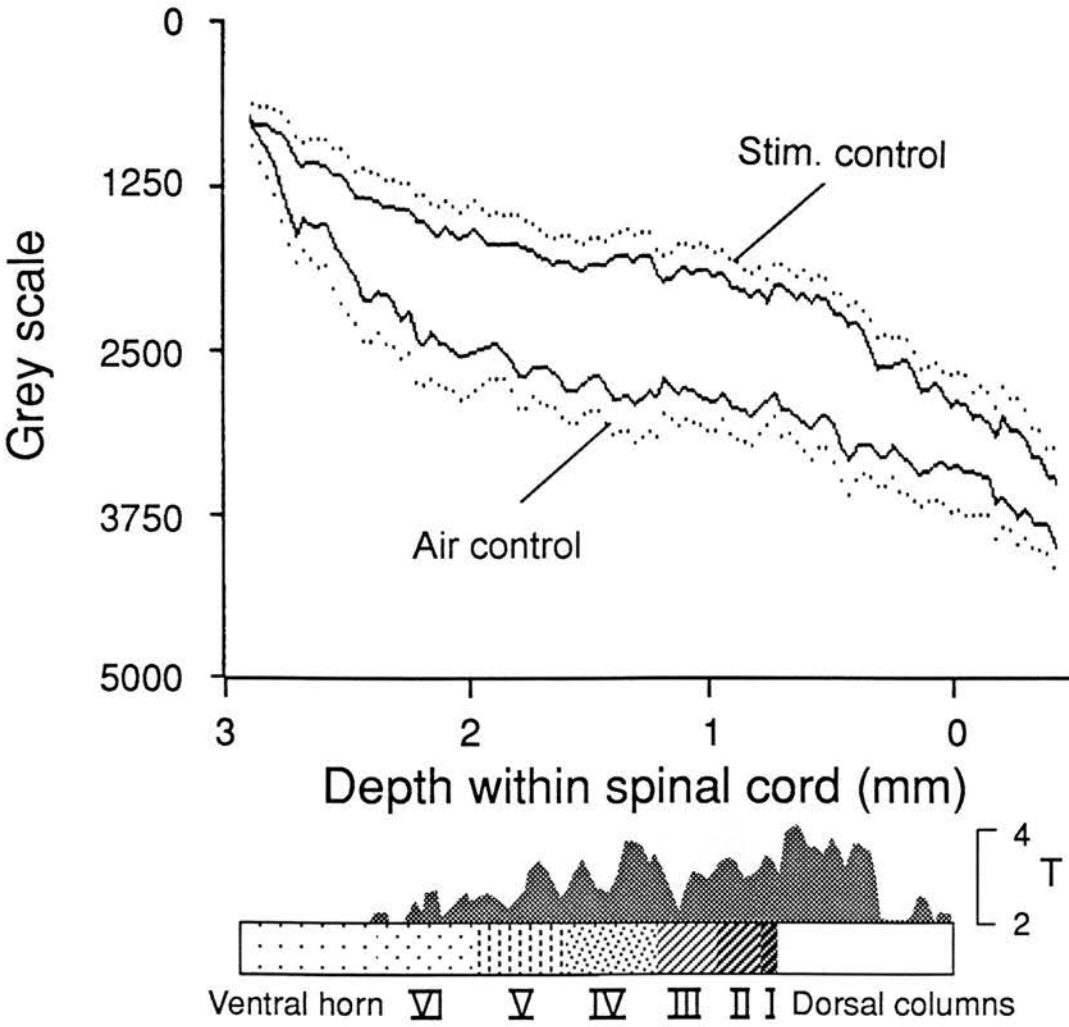
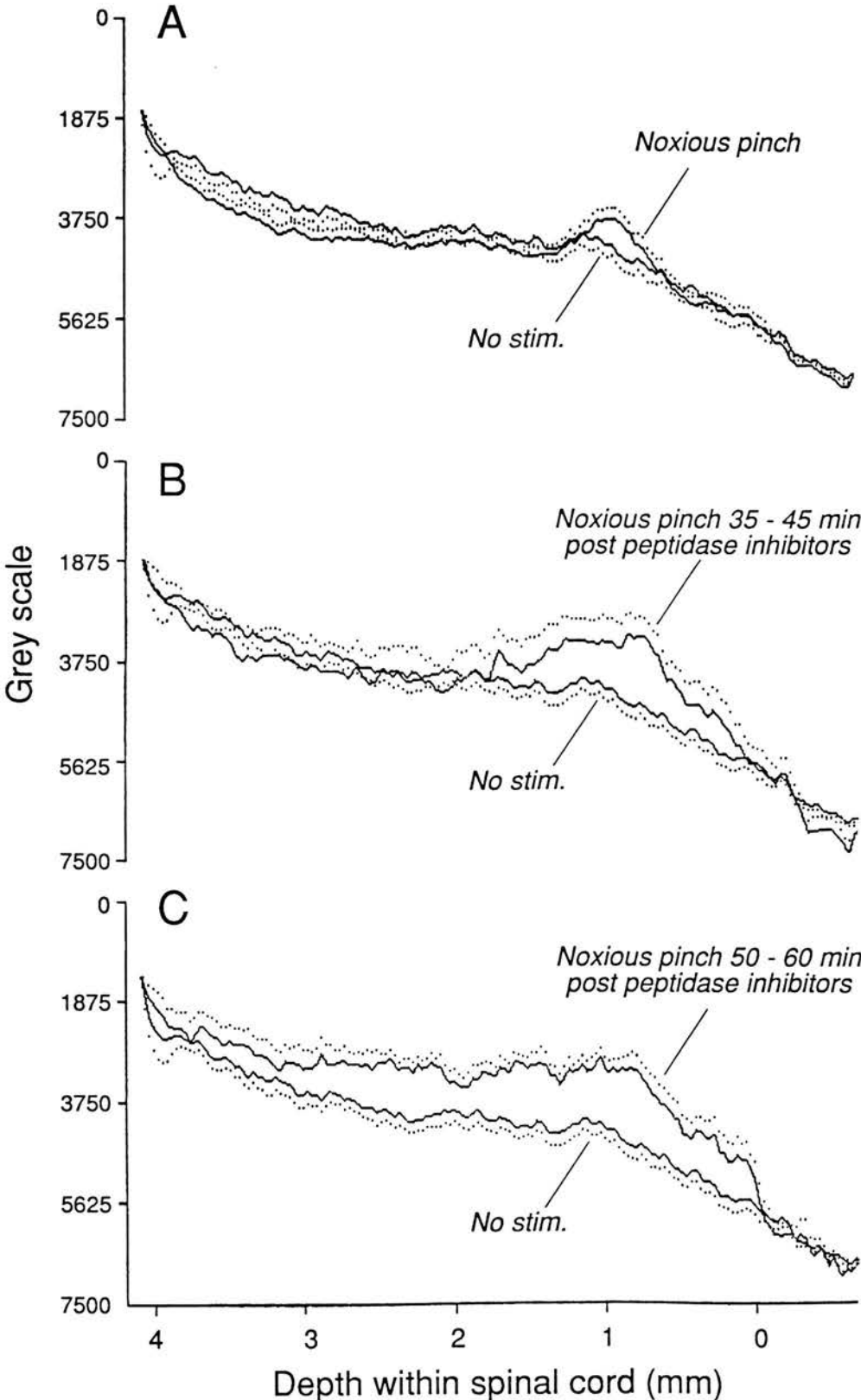


FIGURE 10. Effect of the microinjection of synthetic peptidase inhibitors (kelatorphan and enalaprilat) at 2500, 2000, 1500 and 1000 μ m below spinal cord surface (and in the area of subsequent antibody microprobe insertion) on stimulus-evoked levels of immunoreactive substance P in the barbiturate anaesthetised cat. Microprobes were inserted into the spinal cord for 10 minutes in all cases. Groups of antibody microprobes illustrated are: 'No stim.' (in all figures, n=16) a group of microprobes inserted in the absence of any concurrent noxious stimulus, 'Noxious pinch' groups of antibody microprobes inserted during concurrent noxious pinching of the glabrous skin and interdigital areas of the ipsilateral hind paw either **A.** prior to (n=11), **B.** 35-40 minutes after (n=14) or **C.** 50-60 minutes after (n=9) microinjection of peptidase inhibitors into the dorsal spinal cord. The stimulus evoked pattern of substance P detection clearly changes, in a time dependant manner, following peptidase administration and after one hour resembles the distribution normally seen for stimulus-evoked levels of neurokinin A. Microinjection of CGRP into the dorsal spinal cord has a similar effect on the pattern of substance P detection in the spinal cord (Schaible et al, 1992)

FIGURE 10.
THE EFFECT OF PEPTIDASE ADMINISTRATION ON THE
PATTERN OF SUBSTANCE P DETECTION.



3) irSP persistence following the cessation of an adequate peripheral stimulus has not been systematically investigated , but it appears to be less than that seen with irNKA.

4) Basal levels of irSP in the absence of prior peripheral noxious stimulation are not consistently observed, and where present are much lower than those seen for irNKA.

Stimulation of arthritic joints.

NKA. Following injection of the stifle joint with kaolin/carrageenan, irNKA detection was immediately increased in the spinal cord following flexion of, or application of pressure to, the treated joint at levels which in the untreated animal would be non-noxious. The pattern of irNKA detection was similar to that seen with other forms of stimulation in the normal animal.

SP. Following joint injection with kaolin/carrageenan, joint flexion or application of pressure to the joint at previously innocuous levels, evoked irSP release only after a delay of 6-7 hours. When irSP release was evoked the pattern of detection was different to that seen when applying conventional peripheral noxious stimuli to the normal animal. Stimulation of an arthritic joint resulted in a comparatively broad zone of irSP detection extending ventral to the substantia gelatinosa and dorsally as far as the cord surface. A similar change in the pattern of irSP detection has been reported following intraspinal injection of peptidase inhibitors or calcitonin gene-related peptide (Duggan et al, 1992, Schaible et al, 1992). These observations may be related; developing arthritis may promote the central protection of SP from enzymatic degradation.

1.12 MODULATION OF TACHYKININ RELEASE
FROM PRIMARY AFFERENT TERMINALS.
STUDIES WITH ANTIBODY MICROPROBES.

An inhibition of neurotransmitter release from the central terminals of nociceptors (presynaptic inhibition) has been suggested as being important in several mechanisms of analgesia. Melzak and Wall (1965) proposed a segmental presynaptic inhibitory mechanism activated by impulses in larger diameter (non-nociceptive) primary afferent fibres. Jessell and Iversen (1977) suggested that endogenous compounds, such as met-enkephalin, known to be analgesic at the level of the spinal cord, might act by presynaptically inhibiting primary afferent transmitter release in the spinal dorsal horn. A similar mechanism has been proposed for the supraspinal control of nociceptive processing in the spinal cord, either directly via descending inhibitory fibres or indirectly via activation of segmental inhibitory fibres (Duggan, 1985).

The spinal analgesic effects of certain groups of drugs, including the opiates and the α_2 adrenoceptor agonists, are frequently explained by their proposed activation of such pre-synaptic inhibitory mechanisms operating at primary afferent terminals of nociceptive origin. As outlined in this section, the tachykinin peptides SP and NKA are closely associated with this type of primary afferent terminal, and the pattern of their release in the spinal cord in response to defined peripheral noxious stimuli can now be visualised using the antibody microprobe technique. The work presented in this thesis was performed with the purpose of establishing what contribution, if any, presynaptic inhibition of SP

or NKA release makes to the spinal analgesia produced by various compounds. Drugs already in clinical use (**morphine**, **noradrenaline**, and **medetomidine** an imidazoline derivative α_2 adrenoceptor agonist) and also the neuropeptide **NPY** were evaluated in this way. The results for each drug will be presented in a separate section.

1.13 INHIBITION OF TRANSMITTER RELEASE

FROM PRIMARY AFFERENT TERMINALS:

GENERAL CONCEPTS.

There are several mechanisms (see also **figure 11**) by which a systemically or topically administered compound might bring about a reduction in neurotransmitter release from primary afferent terminals in the spinal cord;

1) activation of fibres of supraspinal origin constituting descending inhibitory controls on spinal cord functions.

2) direct activation of inhibitory systems within the spinal cord itself.

3) a direct inhibitory effect on peripheral receptors or afferent nerve fibres.

1) Supraspinal inhibitory pathways. Electrical or chemical stimulation of many areas of the brainstem results in inhibition of transmission of impulses in neurones of the spinal cord (Carstens et al, 1980, Gernart et al, 1980, Hall et al, 1982, McCreery et al, 1979). A wide variety of neuroactive compounds are contained in these descending inhibitory pathways, including noradrenaline, 5HT, dopamine, SP,

thyroid releasing hormone, cholecystokinin, galanin, NPY, beta-endorphin and enkephalin (see Duggan and Weihe, 1991, for a full review). Similarly, the anatomical organisation of these systems is complex. For example noradrenergic descending inhibitory fibres arise largely from the Kolliker-Fuse nucleus (Stevens et al, 1982) but also from the A6 cell group of the nucleus locus coeruleus, the A7 cell group of the subcoeruleus, parabrachial nucleus and the A5 cell group lateral to the inferior olive (Kwiat and Basbaum, 1992, Westlund et al, 1984). To quote from this last study:

" Our present lack of understanding may be partially explained by the as yet unknown microanatomical relationships of noradrenergic neurones to other classes of cells. Based on anatomical considerations alone, conflicting physiological and pharmacological data can be attributed to the influence of non-noradrenergic systems or to the coactivation or inhibition of two noradrenergic systems which influence areas of contradictory function."

This is a rather protracted way of saying that the mechanisms by which these descending fibres exert their effects in the spinal cord, and the ways in which the different descending inhibitory systems interact are not understood. Despite having been made over ten years ago, the statement still holds true. Descending inhibitory fibres may directly contact neurones involved in primary afferent transmission, or they may contact intrinsic neurones of the spinal cord which in turn influence afferent (or efferent) transmission (see below). In fact both mechanisms could be active. This gap in the understanding of descending inhibitory pathways is unimportant in regard to the present studies, with two exceptions.

The first exception is when a drug is administered in such a way as to stimulate both descending inhibitory pathways and intrinsic spinal mechanisms simultaneously. This could theoretically apply to

the study of the effects of systemically applied morphine on NKA release from primary afferent terminals. Morphine applied intracerebro-ventricularly has been proposed as activating descending serotonergic and noradrenergic pathways inhibiting the transmission of nociceptive impulses in the spinal cord (Suh and Tseng, 1990, Yaksh, 1985). Morphine is also analgesic when administered at the level of the spinal cord (as will be fully discussed in section 3). Systemic administration of morphine to spinally intact animals may activate both mechanisms of analgesia (Yeung and Rudy, 1980) making the contribution of opioid receptor activation in the spinal cord itself difficult to determine. This is one of the reasons why the animals used in the present studies were spinalised at the spino-thoracic level; to interrupt any descending inhibitory pathways.

The second problem arises from the tonic inhibition of spinal cord function (this may be a general phenomenon, rather than selective for particular forms of afferent transmission) mediated by descending inhibitory pathways during surgical anaesthesia (Hall et al, 1981,1982). This is again obviated by spinal cord transection. It is, however, worth noting that the results of one study suggest that there is no tonic descending inhibitory control of irSP release in the anaesthetised cat (Duggan et al, 1988c).

2)Activation of intrinsic spinal cord mechanisms. It might first be noted that in the present studies it is not possible to relate the effects of the drugs used to a particular endogenous control system. Thus, if a spinally-administered compound were to reduce the amount of a particular peptide released from primary afferent terminals as detected by antibody microprobes, it might bring about that reduction

either by a direct action on those primary afferent terminals or by activation of segmental inhibitory neurones of the spinal cord.

As already discussed, receptors located on the central terminals of nociceptive afferents might be contacted in vivo by processes of either descending inhibitory fibres or by intrinsic neurones of the spinal cord. Descending inhibitory fibres have already been discussed. Intrinsic neurones of the spinal cord contain literally dozens of potential neurotransmitters (see Duggan and Weihe, 1991), most of which have not been functionally evaluated. Gamma-aminobutyric acid (Duggan and Foong, 1985) and endogenous opioid peptides (Catley et al, 1983, Duggan et al, 1985) are classically associated with segmental inhibitory systems in the spinal cord, but their exact mechanism of action in blocking primary afferent transmission is not yet known.

The organisation of inhibitory mechanisms within the spinal cord is probably very complex and interactions between more than one system can be observed. For example, in spinally intact rabbits, an interaction between segmental opioid and descending noradrenergic inhibition of an electrically evoked sural nerve reflex has been revealed (Clarke et al, 1988,1989). This type of interaction between opiate and noradrenergic inhibitory systems can also be demonstrated in a number of experimental models involving rodents and the cat (Hylden et al, 1991, Omote et al, 1991, Ossipov et al, 1989, 1990a,b,c, Roerig et al, 1992, Sullivan et al, 1987, Taiwo et al, 1985, Wilcox et al, 1987). Development of cross-tolerance between the two systems has also been shown (Kalso et al, 1993, Solomon and Gebhart, 1988a). However, as with the various descending inhibitory pathways, the functional significance of the various segmental

controls in the spinal cord has not yet been clarified. The actual existence of presynaptic contacts to primary afferent terminals has been established in only a very few cases, and the histological evidence to support a presynaptic mechanism for the inhibition of transmitter release by particular compounds from such sites will be discussed separately for each study.

3) Peripheral effects. At very high doses (much higher than those used in the present study) morphine may block the propagation of action potentials in primary afferent fibres (Jurna and Grossman, 1977). The sensitisation of peripheral receptors during the processes of tissue damage and inflammation has previously been discussed. There is some evidence that peripheral (in many cases articular) opiate receptors may in part mediate the analgesia produced by morphine in such inflammatory states (Khoury et al, 1990, Parsons et al, 1990, Russell et al, 1987, Stein, 1993, Stein et al, 1990a,b). Other drugs such as aspirin (Grubb et al, 1991, Guilbaud and Iggo, 1985, Heppelmann et al, 1986, Monkada et al, 1975) and serotonin (Birrell et al, 1990) have been shown to desensitise articular receptors in experimental models of arthritis, and such desensitisation will presumably lead to a reduction in the amount of neurotransmitter released from the central projections of such receptors under these conditions.

FIGURE 11. Possible sites of action for drugs leading to altered spinal processing at the first afferent synapse.

Drugs may activate descending or segmental inhibitory fibres at their source (A) or may mimic the effects of the endogenous neurotransmitters which such fibres release: either postsynaptically (B) on second order neurones in the spinal cord or presynaptically (C) on the synaptic terminals of primary afferent fibres. This effect may not be direct but may mimic the effects of neurotransmitters released by spinal interneurons activated by such descending/segmental pathways (D,E). Alternatively drugs may have direct effects on primary afferent terminals or their synaptic contacts unrelated to endogenous control mechanisms (F,G) or modulate pre- or postjunctional effects at the synapses between descending/segmental fibres and spinal interneurons (H,I). Lastly drugs may alter the transmission of nociceptive information in the primary afferent fibres themselves (J) or alter transduction mechanisms in receptors located in peripheral tissues (K).

SECTION 2

GENERAL METHODOLOGY

2.1 STORAGE OF PEPTIDES AND OTHER ORGANIC COMPOUNDS.

A large number of chemicals are used in the preparation and use of antibody microprobes. Most of these are inorganic and their handling and storage is straightforward. The organic compounds are not so robust and certain precautions were taken in their use.

Antibodies.

All antibodies were purchased commercially, and were delivered as freeze-dried lyophilates. In some cases this lyophilate was stored at -20°C for several weeks prior to reconstitution, without any apparent loss in the integrity of the antibody. These lyophilates already contain buffering salts and bovine serum albumin (BSA) resulting in the addition of a minimum volume of diluent being required to fully dissolve them completely. In practice it was found that all of the antibodies used could be reconstituted at double the manufacturers suggested concentration for radioimmunoassay (RIA). Lyophilates were reconstituted in distilled and Millipore filtered water and the resulting solution was immediately divided into 0.5ml aliquots in capped, polythene Eppendorf tubes. These aliquots were then stored at -20°C (in later studies at -70°C) until use. As with all organic solutions, the unused portion of any thawed aliquot of antibody was discarded, as re-freezing promotes the breakdown of such compounds.

The absolute number of antibody molecules in a given volume of solution is not determinable when purchasing such commercial antisera designed for use in RIA studies. However, one batch of antisera was sufficient for 15 to 25 antibody microprobe experiments, and the

concentration of antibody used in any one study was constant. Furthermore, the suitability of each new batch of antisera for use in microprobe experiments was tested by a variety of in vitro tests, as will later be described

Unlabelled peptides.

These were purchased in freeze-dried form and reconstituted according to the suppliers recommendations with the appropriate diluent. Peptides were initially reconstituted to various concentrations and stored as 100 μ l aliquots in capped Eppendorf tubes at -20°C. The bulk of the peptide was stored as a 10⁻⁵M solution, which was later used to make up batches of 10⁻⁶, 10⁻⁷ and 10⁻⁸M aliquots, these then being stored in a similar fashion.

Radiolabelled peptides.

Commercially ¹²⁵I-labelled SP and NKA were purchased from Amersham, UK in batches, each containing a total of 10 μ Ci of radioactive material. Each 10 μ Ci batch was reconstituted immediately on delivery with the recommended diluent to a total volume of 100 μ l and then divided into 20 x 5 μ l aliquots. These aliquots were stored in a dedicated, lead screened freezer at -20°C until required. Following thawing, single aliquots were diluted in a solution of phosphate buffered saline (PBS)/azide containing 0.5% BSA to give a solution with a gamma emission of 2000 counts minute⁻¹(cpm) μ l⁻¹. This final dilution was made only on the day of use, and the solution was then stored on ice (but not frozen) until required. ¹²⁵I has a half life of 60 days, and unused aliquots of radiolabelled peptide were discarded 6 weeks after their specified activity date.

2.2 ANTIBODY MICROPROBE PREPARATION.

I. SILOXANE POLYMER COATING.

The technology of antibody microprobe production is not static, that is to say that refinements in the process are constantly being tested and applied. Consequently, the preparation of antibody microprobes has changed in various subtle ways since the work presented in this thesis was initiated. What is presented here is the current method of antibody microprobe production. The techniques have most recently been reviewed by Duggan, 1992. The processes involved in antibody coupling to the glass were illustrated earlier in **figures 4 and 5.**

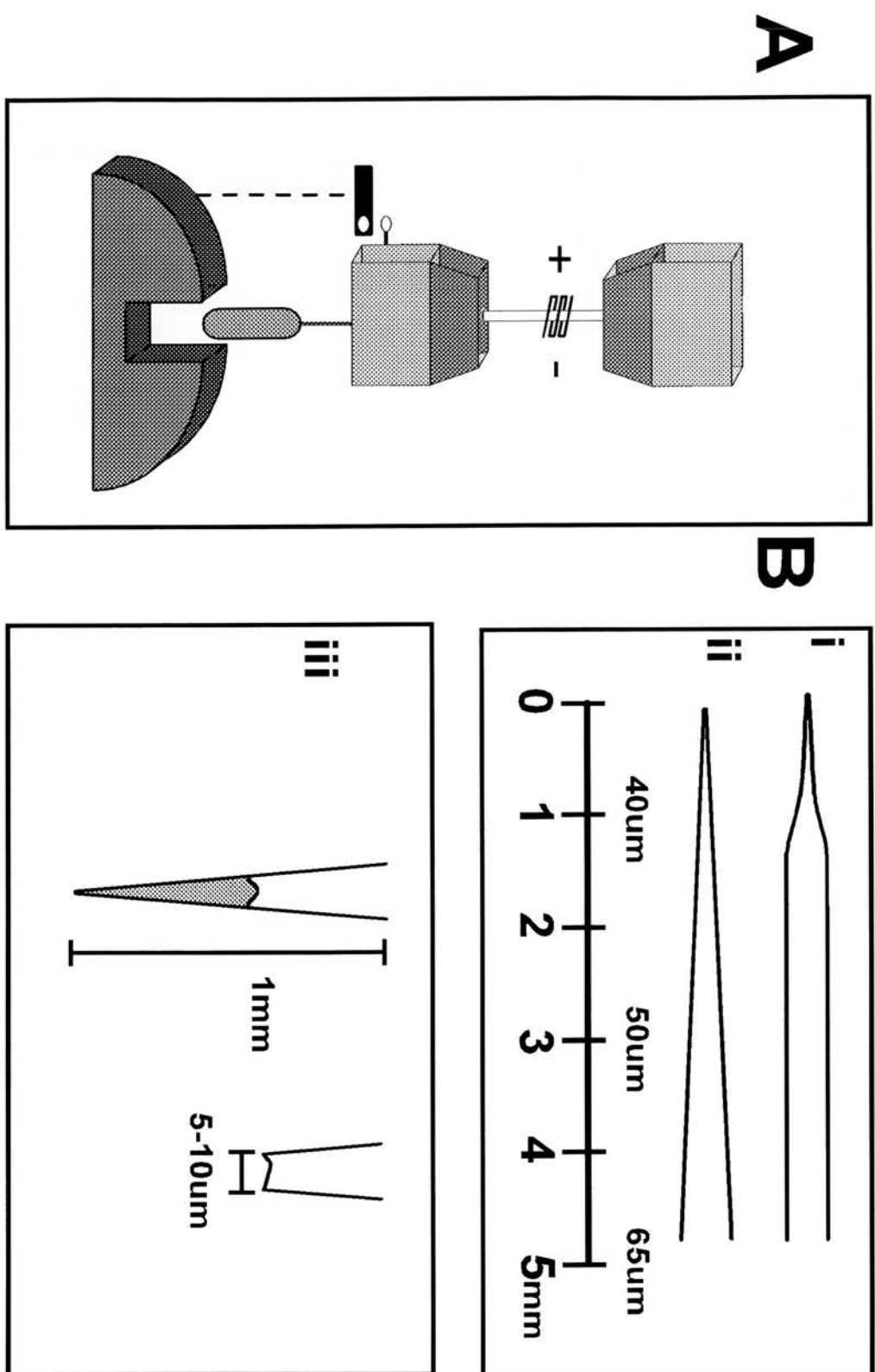
Preparation of glass.

Hollow Pyrex glass tubing (outer diameter 3mm, inner diameter 2mm, non-filament) of the type commonly used to make electrophysiological recording electrodes was used to manufacture the antibody microprobes. The glass was drawn out by heating it in the coil of a microelectrode puller (Clarke), with the settings adjusted to give an even taper from tip to shank (see **figures 12A and 12B**). Both ends of the microprobe were then heat sealed; the wide, unpulled end in a gas flame and the tip by touching it briefly against a heated coil, controlled by a micromanipulator and placed under a binocular microscope to allow accurate sealing. Approximately 100µm of the tip was sealed by this method. The resultant glass microprobe is very flexible and resistant to accidental breakage, both factors being requisite for their subsequent use. The microprobes were then

FIGURE 12. A. Outline of mechanism for pulling glass microprobes. A hollow tube of pyrex glass is suspended between two holders, running through a heated metal coil at midlength. The lower holder is attached to a metal weight. As the glass is heated it stretches and the lower holder drops, until, at a particular distance a switch is activated, which triggers a strong electromagnetic field, rapidly pulling the weight down and drawing the molten glass out to a point. All factors (heat of coil, drop until pull triggered and pull force) are variable allowing fine adjustment of microprobe shape.

B. Typical dimensions of antibody microprobes. Electrodes for physiological recording (i) tend to have an abrupt taper towards the end. Antibody microprobes (ii) have a more even taper with the dimensions shown. Antibody microprobes are stored with the tips heat sealed (iii) and these sealed tips must be broken off before in vivo use leaving a tip diameter of 5-10 μ m as shown.

FIGURE 12.
ANTIBODY MICROPROBES: SHAPE AND DIMENSIONS.



placed in glass carrying buckets in groups of approximately twenty for ease of handling. Microprobes, in buckets, were immersed in 10% nitric acid for 30 minutes and then given 3 x 10 minute washes in Millipore filtered distilled water. The buckets of probes were then dried in a clean oven at 200°C for at least 2 hours, or until further use. This process resulted in clean, dry microprobes with some degree of surface etching on which subsequent chemical reactions could proceed.

Preparation of reagents.

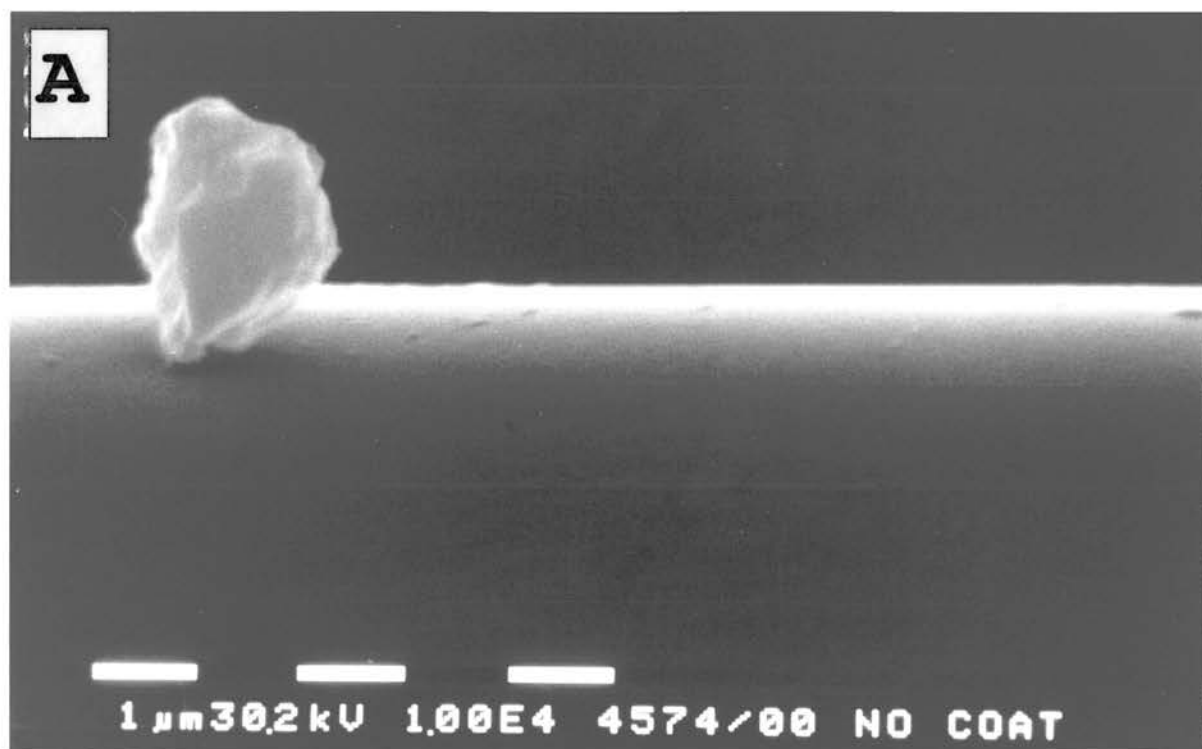
Glass carrying buckets of the same type used to hold the microprobes were filled with molecular sieves (Aldrich 4A, 4-8 mesh) and heat dried at 200°C for 2 hours. Clean Pyrex-glass boiling tubes (Quickfit 24/29) were heated to 200°C and then allowed to cool. Whilst still warm they were placed in a fume cupboard and filled with 10ml aliquots of gamma-aminopropyltriethoxysilane (APTES) (Aldrich) taken from a freshly opened 100ml bottle (it was usual to prepare 8 such tubes in each session of microprobe production). Unused APTES is discarded. To each boiling tube 40ml of reagent grade toluene (Aldrich) was then added, and the tubes were stoppered with polythene caps. The buckets of molecular sieves were removed from the oven and allowed to cool slightly. One bucket of sieves was then added to each tube of APTES/toluene mixture. The tubes were transferred to a humidity cabinet saturated with water vapour at room temperature and left for 1 hour. The purpose of the molecular sieves was to remove as much water as possible from the APTES/toluene mixture, as excessive water in the solution will interfere with the even build up of siloxane polymer on the surface of the microprobes.

2.Exposure of glass to reagents.

The buckets of glass microprobes were carefully removed from the oven with metal forceps and suspended in the humidity cabinet for 1 minute, allowing a fine layer of water vapour to adsorb to their surface. The presence of at least a small amount of absorbed water on the surface of the glass was required for the initiation of the formation of siloxane polymer. As quickly as possible the boiling tubes were then opened, the buckets of molecular sieves removed and the buckets of microprobes lowered into the APTES/toluene mixture. Each tube of reagent, now containing a bucket of microprobes was then restoppered and removed from the humidity cabinet. Boiling tubes were then placed in a centrifuge and spun at 6°C, 2000rpm for 1 hour. This centrifugation resulted in a more even build up of siloxane polymer on the surface of the microprobes. This may have been due either to centrifugation of free water away from the tips of the microprobes or to the removal of small particles of debris from the solution around the tips which may act as foci for the polymerisation reaction. The stoppered tubes were then removed from the centrifuge and left at room temperature for 12 hours. The buckets of microprobes were then removed from the boiling tubes in a fume cupboard and washed by brief immersion in toluene, after which they were allowed to dry at room temperature before being placed in an oven at 200°C for 24 hours to cure the siloxane polymer. The APTES/toluene mixture was then discarded.

FIGURE 13. Scanning electron micrographs of **A.** the surface of an uncoated glass microprobe and **B.** the surface of a siloxane coated microprobe. (Both x20,000).

FIGURE 13.
ELECTRON MICROGRAPHS OF SILOXANE-COATED AND
UNCOATED MICROPROBES.



3. Assessment of siloxane coating.

Scanning electron micrographs of the surfaces of both uncoated and siloxane coated microprobes are illustrated in **figure 13**. It can be seen that the polymer coating not only provides a chemical base for the subsequent immobilisation of antibodies to the microprobe but also vastly increases its surface area. A good polymer coat on the surface of the microprobes will appear, under x40 magnification using incident light, as a milky deposit on the surface of the microprobes; white prior to heat curing and yellowish afterwards. The microprobes were subsequently used to detect peptide binding at a resolution of 50-100 μ m, and consequently assessment of polymer coating in this way was not sufficiently accurate. Following curing, individual microprobes were placed in a micromanipulator mounted under a binocular microscope and examined at x125 magnification using transmitted light. This allowed the siloxane coating to be examined in great detail.

It was important to identify microprobes with uneven or patchy distribution of siloxane coating, which were discarded, and also to identify microprobes with small focal aggregations of polymer. In some cases these 'lumpy' microprobes could be made usable by wiping the tip vigorously with soft tissue; this tends to remove irregular lumps of siloxane polymer whilst leaving the underlying layers intact. Up to 60% of microprobes were routinely discarded at this stage, leaving only those with a very even coating of siloxane polymer. Microprobes designated adequate for in vivo use were then stored horizontally in specially prepared grooved metal racks, inside covered boxes, until required. Microprobes were sorted into three groups; those with light, medium and heavy siloxane polymer coatings.

Although the analysis of microprobe images (see later section) is performed in such a way as to minimise the effects of coat density on the final result, it was felt that each experiment should utilise microprobes with similar densities of siloxane, and consequently of antibody, coating.

2.3 ANTIBODY MICROPROBE PREPARATION.

II.ANTIBODY COUPLING.

The binding of antibodies to siloxane coated microprobes takes from 3-5 days and the use of the antibody microprobes must be timed to prevent their wastage. Siloxane coated microprobes can be stored indefinitely, but once organic molecules have been linked to the microprobe coating they have a relatively short lifespan. Unpublished in vitro work suggests that, following completion of antibody coupling, the microprobes will lose approximately 25% of their binding affinity for the relevant peptide every 7 days. Microprobes were, therefore, used within 24 hours of their preparation.

There are three stages in the coupling of antibodies to the siloxane coated microprobes, each stage being designed to maximise both the specificity and affinity of the finished microprobes for the peptide of interest.

1.Glutaraldehyde

Aldehyde groups are utilised for the subsequent immobilisation of proteins to the siloxane polymer. Siloxane coated probes were selected (and their coatings given a final check under the

microscope) in the desired numbers for the proposed experiments. Microprobes were placed in groups of 20 in the handling buckets previously mentioned and immersed in a 2.5% solution of glutaraldehyde in distilled water for 30 minutes. The buckets of probes were then washed for 3 x 10 minute washes in distilled water before proceeding immediately to stage 2.

2. Glutaraldehyde/Protein A coupling.

Protein A is derived from culture of *Staphylococcus aureus* and has a high selectivity for binding immunoglobulins of the IgG class. Protein A (Sigma) was diluted in PBS containing 0.1% sodium azide, to a final concentration of 0.1mg ml^{-1} . 5 μl glass microcapillaries were filled with the protein A solution. Under a dissecting microscope, the tip of each glutaraldehyde treated microprobe was then placed into one of the protein A filled capillaries. In any case where the sealed tip of an antibody microprobe was broken at this stage, that microprobe was discarded. Each microprobe with its tip inside a capillary was then placed horizontally on a perspex rack. The rack was placed in a covered plastic tray containing, in its base, tissue paper soaked in PBS/azide. This fluid was to prevent loss of the protein A solution from the capillaries by evaporation during the subsequent incubation. The covered racks were then placed in a cold room at 4°C for 24 hours.

3. Protein A/Antibody coupling.

Following incubation in protein A, microprobes were removed from their capillaries and placed in petri dishes containing sodium borohydride 2.5% w/v in borate buffer. The ensuing reaction, which

produces free hydrogen, reduces the Schiff bases which may form by aldehyde coupling of amino groups on the protein A. The microprobes were then replaced in the handling buckets and given 3 x 10 minute washes in PBS/azide solution. Commercially obtained polyclonally derived antisera to either SP or NKA (Peninsula Laboratories) was diluted to twice the manufacturers recommended dilution for radioimmunoassay. (Ideally antibody would have been used in an even more concentrated form. Commercially prepared antisera such as these are supplied, however, as a lyophilate which already contains buffering salts and antibacterial agents making its reconstitution in more concentrated form impossible.) 5 μ l glass capillaries were filled with the required antibody solution and the tip of each microprobe was inserted into a capillary as previously described. The microprobes were again placed on perspex racks in covered boxes and incubated at 4°C for 24-48 hours.

A few hours before their use in vivo, the capillaries were removed from the antibody coated microprobes which were then placed in glass handling buckets, and stored ready for use immersed in PBS/azide solution.

2.4 IN VITRO TESTING OF ANTIBODY MICROPROBES.

Before use in vivo, antibody microprobes were subjected to numerous in vitro tests of their selectivity and affinity for the ligand being studied. This in vitro testing was carried out in two stages 1) extensive in vitro checking of antibody microprobes prepared with a newly purchased batch of antibody, prior to planning any in

vivo work with that antibody and 2) a more intensive check of microprobe function concurrent with each in vivo experiment.

When purchasing a new antibody attention was paid to the manufacturers claims regarding the cross-reactivity of the antibody with other peptides. Thus, for the C-terminally directed antibody to SP used in two of these studies, Peninsula Laboratories stated that the antibody cross-reacted with fragments of SP containing at least the last 5 amino acid residues, but showed negligible cross reactivity with NKA and NKB. The C-terminally directed NKA antibody, again from Peninsula Laboratories, was stated as having 100% cross-reactivity for NPK (the N-terminal extended form of NKA), 70-80% cross reactivity with NKB and negligible cross reactivity with SP. This was the most selective NKA antibody commercially available at that time. Given that SP and NKA are released in the same area of the spinal cord and in response to the same forms of peripheral stimuli, it was important to verify the manufacturers claims for antisera specificity prior to commencing in vivo work.

1) Newly purchased antibody.

A number of antibody coated microprobes were prepared as previously described. These were divided into groups of approximately six and marked with indelible marker pen to allow their subsequent identification. These microprobes were then inserted into 5 μ l capillaries filled with various peptide solutions, and incubated for 30 minutes at 37°C in a humidified box (to prevent evaporation of the peptide solutions from the capillaries). Taking microprobes coated with antibody to SP as an example, the solutions used might optimally be as follows;

Group 1	no incubation	n=6
Group 2	10^{-6}M SP	n=6
Group 3	10^{-7}M SP	n=6
Group 4	10^{-8}M SP	n=6
Group 5	10^{-7}M NKA	n=6
Group 6	10^{-7}M Dynorphin (1-8)	n=6

Following their incubation in peptide the microprobes were mounted in a holder and lowered into a chilled solution of PBS containing 0.1% Tween, stirred magnetically. The tips of the microprobes were left in this solution for 15 minutes to remove any unbound peptide from their surfaces. After washing, the tips of the microprobes were placed in 5 μl capillaries filled with a solution of Bolton-Hunter labelled ^{125}I -SP, diluted in PBS/azide containing 0.5% BSA to give 2000 counts $\text{minute}^{-1} \mu\text{l}^{-1}$. BSA was added at this stage to block non-specific tracer binding to the microprobe surface. The microprobes were then incubated in the capillaries filled with tracer solution for 24 hours at 4°C.

Following incubation in the radiolabelled tracer solution the microprobes were removed from the capillaries and each microprobe had both sealed ends removed; the thick end with a glass saw and the tip with watchmakers forceps under a dissecting microscope. As short a length of the tip as possible was removed in this way. The probes were again washed for 15 minutes in chilled PBS/Tween. This time, however, the thick ends of the microprobes were mounted in a sealed perspex block which was in turn connected to a vacuum pump. Probes were washed under suction at this stage to suck the washing solution through the inside of the microprobes, thus removing any

radiolabelled peptide which might have been drawn in during the preceding incubation.

The tips of the microprobes were then broken off, between a thumb and forefinger, to a uniform length of approximately 1.5 cm. The tips were then fixed to narrow strips of cardboard with a small amount of liquid paper (Tipex) applied to the thick end. The strips of card were numbered to allow subsequent identification of the individual microprobe tips. These strips of card were then placed in Beckmann RIA gamma-counter tubes, and the amount of radioactivity, in counts minute⁻¹, was determined for each tube. (An example of typical counts obtained in this way, but for a less extensive in vitro test, is illustrated in **table 4**). Lastly, the strips of card bearing the microprobe tips were fixed to a sheet of cartridge paper with Tipex. This sheet of paper was placed in a standard (non-screen) x-ray film cassette with a sheet of monoemulsion x-ray film (Kodak NMB) and left for several days to produce autoradiographic images of the microprobes. The protocol for testing anti-NKA coated microprobes in vitro is essentially the same, but obviously using radiolabelled NKA and with the substitution of NKA solutions for SP solutions and vice versa.

TABLE 4. The results of a typical in vitro test of antibody microprobe function. The table is, in fact, the printout from a Beckmann RIA gamma counter, used to measure the ^{125}I -substance P binding of antibody (substance P) microprobes after incubation in a variety of solutions.

Tubes, 1,2,3,	Saline (controls)	No suppression of binding.
Tubes, 4,5,6,	Substance P (10^{-6}M)	>75% suppression.
Tubes, 7,8,9,10,11	Galanin (10^{-6}M)	No statistically significant suppression.
Tubes, 12,13,14,15,16,	Substance P (10^{-7}M)	>50% suppression.

TABLE 4.

TYPICAL RESULT OF AN IN VITRO TEST OF ANTIBODY
MICROPROBE FUNCTION.

	PARAMETERS	01	
01	ID	:I-125	→
02	TIME-S (SEC)	0060	→
03	TIME-U (SEC)	0060	→
04	IS (I-125)	01	→
05	METHOD	06	→
06	REPLICATES	01	→
07	PRINTOUTS		1.2.3.5.6.8.
08	FACTOR 1	+1.000E+02	→
09	FACTOR 2	+6.000E+01	→
10	FIRST PAT.NO	0000	→
11	FIRST PAT.NO	0001	→
12	POS-CODE	0000-	→

PAT	DET	POS	TIME	COUNTS1	COR.CPM1	MEAN
0	1	1	60	1932	1899.4}	
0	1	2	60	2097	2065.4}	2019.4
0	1	3	60	2125	2093.5}	
0	1	4	60	437	396.2}	
0	1	5	60	453	412.3}	351.6
0	2	6	60	272	246.2}	
0	2	7	60	2703	2709.8}	
0	2	8	60	1348	1336.6}	
0	2	9	60	1725	1718.7}	1643.0
0	2	10	60	619	597.9}	
0	3	11	60	1851	1852.4}	
0	3	12	60	445	419.0}	
0	3	13	60	717	696.3}	
0	3	14	60	791	771.7}	687.2
0	3	15	60	1012	997.0}	
0	3	16	60	627	552.0}	

Several conclusions can be drawn from the results of such an assay, the different groups giving different information as follows.

Group 1: the amount of bound tracer on these microprobes gives an indication of the **affinity** of the microprobe for the given ligand. This may be influenced by the actual amount of antibody bound to the probe surface or by the binding affinity of the particular antibody used, and this may vary slightly from batch to batch. Anti-SP coated microprobes generally gave unsuppressed radioligand binding in the range of 1500-2000 cpm, anti-NKA coated microprobes tended to show a slightly lower level of radioligand binding, in the region of 1000 cpm.

Groups 2-4: these groups give an indication of the **sensitivity** of the microprobe assay and its suitability for in vivo application. Prior incubation in an unlabelled peptide, for which the antibody shows affinity, reduces the number of antibody binding sites available for subsequent radioligand binding. The total amount of tracer binding is, therefore, suppressed. The exact relationship between the in vitro sensitivity and in vivo sensitivity of a microprobe cannot be determined, as the concentrations of peptide at the sites of release in the spinal cord are not determinable. All previous microprobe studies on tachykinin release in the dorsal horn of the spinal cord have, however, shown that where radiolabelled tracer binding is suppressed by 50% or more by prior incubation in a 10^{-7} M solution of unlabelled ligand, microprobes will be effective in detecting release in vivo. In all studies presented here, the suppression of tracer binding by 10^{-7} M ligand was in this range. In

fact, in some tests, suppression by the 10^{-8}M peptide solution was greater than 50%.

Groups 5 and 6: These groups give an indication of the selectivity of the antibody microprobes for the selected peptide. Prior incubation in a structurally related peptide (Group 5), verifies the manufacturers claim that the SP antisera shows no cross-reactivity for NKA and vice versa. If this is the case, then no suppression of radiolabelling should occur, and indeed this was the case with all antisera used in these studies. The inclusion of microprobes pre-incubated in a completely non-related peptide (Group 6) strengthens the identification of the antisera as selective for the peptide being studied. Such pre-incubation should not, and in no case did, suppress subsequent radioligand binding.

Authoradiographs. The tips of the in vitro antibody microprobes are evenly exposed to the radiolabelled peptide, so the autoradiographs of the microprobe tips should show an even distribution of tracer binding. Study of these autoradiographs allows a very sensitive assessment of the evenness of the distribution of antibody on the microprobe surface. The siloxane polymer can itself be visualised under the microscope, but the examination of autoradiographs is the only way to monitor the even application of the organic layers to the antibody microprobes. On the rare occasions where the autoradiographic images of in vitro probes had a lumpy or uneven distribution of radiolabelling, a few antibody coated microprobes were incubated in a ^{125}I -labelled antibody raised against the FAb region of the peptide antisera being used. In no instance

were the autoradiographs of microprobes labelled in this way uneven. Where in vitro microprobes produced poor quality images this was, therefore, attributed to a degeneration of the radiolabelled peptide solution which was replaced.

2) In vitro tests concurrent with in vivo experimentation.

During each in vivo experiment, a small number of the prepared antibody microprobes were selected for use in a small in vitro test of antibody microprobe quality. These were divided into two groups and treated in the way described for groups 1 and 3 of the larger in vitro study described earlier in this section, ie. no suppression of tracer binding, and suppression with 10^{-7}M unlabelled peptide. This test was performed to confirm that the microprobe affinities and sensitivities were still as expected from the earlier in vitro investigations. An additional benefit was that the radioactivity counts from the unsuppressed group of in vitro microprobes gave some indication of how long the in vivo microprobes should be left on x-ray film to produce usable autoradiographs.

2.5 ANIMAL PREPARATION.

Barbiturate anaesthetised cats were used in all experiments. Male and female cats in a weight range of 2.4 to 3.2kg, bred specifically for research purposes, were purchased from authorised suppliers (BS+S/Hillgrove). Animals were typically housed for at least one week prior to their use in order to minimise any stress-induced alterations in their physiology which might be brought about by transportation and change of environment.

Induction of anaesthesia was by intraperitoneal injection of sodium pentobarbitone (Nembutal, CEVA Ltd), 35mg kg^{-1} , 35mg ml^{-1} in physiological saline. Throughout surgery, anaesthesia was maintained by bolus injection of the same drug at 10 mg ml^{-1} into one or other cephalic vein. Following the completion of surgery a continuous intravenous infusion of sodium pentobarbitone at 6mg ml^{-1} at a rate sufficient to maintain clinical anaesthesia was administered via cannulation of a cephalic vein for the duration of the experiment. Following induction of anaesthesia cats were given a subcutaneous injection of ampicillin (Norobrittin, Norbrook Laboratories Ltd), 25mg kg^{-1} , which was repeated 10 hours later. This injection of antibiotic was to help maximise the sterility of the experimental preparation, particularly with regard to the subsequent exposure of the spinal cord. Throughout surgery and experimentation the cat was placed on an electrically heated blanket to maintain body temperature at 37.4°C . This was controlled via a rectal temperature probe connected to the blanket control box.

Initial surgery involved the cannulation of 1) a cephalic vein in the foreleg to allow subsequent anaesthetic/drug administration

2) one carotid artery which was then used to continuously monitor systemic blood pressure and 3) the trachea, allowing artificial positive pressure ventilation where required and continuous monitoring of end tidal CO₂ levels (maintained at 4%).

The animal was then placed in ventral recumbency, laterally supported with sandbags, and the skin over the dorsum was incised rostrocaudally by electro-cautery. The connective tissue overlying the muscles of the transversospinalis muscle group was then cut longitudinally either side of the dorsal vertebral processes from the sacro-iliac joint rostrally as far as the third lumbar vertebra. Transversospinalis and longissimus muscles were then retracted laterally from the dorsal surface of the vertebrae by blunt dissection, and held clear by retractors. Any bleeding from muscle capillaries at this stage was stopped by cauterisation.

The dorsal surface of the spinal cord was then removed with rongeurs, from the sacroiliac joint to approximately 1cm rostral to the lumbar enlargement of the spinal cord and the exposed spinal cord temporarily covered with cotton-wool soaked in sterile Ringer's solution. The transversalis muscles on either side of the laminectomy were then removed. Lastly, a second, much smaller dorsal laminectomy was made in the region of the thoraco-lumbar junction. The exposed spinal cord at this site was injected with 0.1ml of a 2% solution of lignocaine (Xylocaine, Astra) and after a few minutes cut with a pair of fine pointed scissors. A small cotton-wool ball soaked in sterile Ringer's solution was then placed over the site of cord transection.

The cat was then transferred to a stereotaxic frame. The preparation was held in place by 1) ear bars, 2) 3 spinal swan-necked clamps placed under the vertebral transverse processes on each side

of the backbone in the area of the spinal lumbar enlargement, 3) a clamp around the vertebral body immediately caudal to the site of spinal cord transection, 4) a clamp placed over the dorsal processes of the sacrum and 5) hip pins placed in the region of the iliac crests. These clamps not only supported the animal in the frame, but also provided excellent spinal cord stability. The cotton-wool covering the exposed cord was removed and, under a dissecting microscope, the edges of the laminectomy were packed with a sterile haemostatic gauze (Sterispon) to prevent subsequent bleeding. The dura mater was then cut with sterile iris scissors and retracted laterally. The exposed cord was finally covered with a thin layer of a sterile solution of agar in Ringer's solution.

At proposed sites of antibody microprobe insertion, a small area of agar was removed with sterile watchmakers forceps to expose the spinal cord. This pool was then continually irrigated with sterile Ringer's solution at 37°C to prevent drying of the cord surface. Watchmakers forceps were also used to remove very small areas of pia arachnoid within the pool, such patches being necessary for subsequent antibody microprobe insertion into the spinal cord.

In experiments in which peripheral nerve stimulation was used, both hind legs were elevated and supported by clamps and the skin overlying the gastrocnemius muscles was cut and stitched to a pool-maker. The tibial nerves were exposed between the muscles of the calf which were retracted laterally. The exposed nerves were mounted (untransected) on platinum stimulating electrodes and the pools were flooded with liquid paraffin. In experiments where electrical nerve stimulation was used, or where artificial ventilation was necessary, animals were given the neuromuscular blocker gallamine triethiodide

(Flaxedil) $4\text{mg kg}^{-1} \text{ hr}^{-1}$ by injection into the cannulated cephalic vein. Thresholds for nerve stimulation were determined before neuromuscular blockade, and in all cases the effects of gallamine were allowed to wear off periodically to allow verification that the level of barbiturate anaesthesia was unaltered.

At the end of each experiment the animal, still anaesthetised, was killed by means of intravenous injection of a concentrated solution of sodium pentobarbitone (Euthesate, Willows Francis Veterinary), which resulted in cardiac arrest within a few seconds.

2.6 IN VIVO USE OF ANTIBODY MICROPROBES.

Prior to the insertion of antibody microprobes into the spinal cord, a NaCl filled extracellular recording electrode placed in the superficial dorsal horn or a silver ball recording electrode placed on the cord surface were used to record field potentials elicited by innocuous cutaneous stimulation of the hind paws. These field potentials were displayed on a cathode ray oscilloscope. Gentle pressure applied to the toes or brushing of the skin of the foot were commonly employed. This recording was made to ensure that the proposed site of microprobe insertion did receive a substantial primary afferent input from the ipsilateral hind paw.

Microprobes, prepared as described earlier, were removed from the PBS/azide solution in which they were stored immediately prior to use and both sealed ends were removed, the thick end with a glass saw and the tip by gently bumping it against the surface of a metal coil under a binocular microscope. The microprobes were examined under

x125 magnification to check the diameter of the broken tip (between 5 and 20 μ m), and the evenness of the siloxane coating. Microprobes were numbered using a coded system of coloured rings, and filled with a solution of 2% pontamine sky blue in sodium acetate. This filling of the microprobes with dye was performed to facilitate microprobe visualisation in subsequent procedures.

One microprobe was then placed in each of two stepping motor micromanipulators, one orientated vertically and the other 10° from vertical. The manipulators were then moved down by hand until they were close to the spinal cord surface. Spinal cord irrigation was temporarily stopped and excess Ringer on the surface of the cord removed by suction. Under x15 magnification, the tips of the two microprobes were moved in the horizontal plane until both overlay the patch which had earlier been made in the pia mater at the proposed site of insertion. Paired microprobes were positioned as close together as possible. The microprobes were then lowered with the micromanipulators until the tips were just touching the surface of the spinal cord. The micromanipulators were zeroed and then used to insert the microprobes into the dorsal spinal cord to a depth of 3.0 or 4.0mm from cord surface (depending on the study). In the case of the micromanipulator angled at 10° from the vertical this meant moving it down to a slightly greater reading eg. 4.25mm.

Once the microprobes were in situ the Ringer irrigation system was turned on again. The length of time that individual microprobes remained in situ, and the peripheral stimuli applied during that time, varied according to the experimental protocol, and will be outlined separately in the methods section for each study. Following exposure to the spinal cord, microprobes were treated in the same as

that earlier described for in vitro tests; washing in PBS/Tween, 24 hour incubation in radiolabelled tracer and a final wash in PBS/Tween with suction. The tips of the in vivo microprobes were not mounted on strips of card, but were stuck directly onto a sheet of cartridge paper with Tipex before this was itself placed in a cassette with monoemulsion x-ray film. The previously assigned microprobe number corresponding to each tip was written on the paper just above it. The exposure time necessary to produce autoradiographs of microprobes suitable for analysis varied from 3 days to 4 weeks. The required time for exposure was estimated by taking into account the amount of unsuppressed radioligand binding on in vitro microprobes. Such in vitro testing was performed concurrently, as previously described, with each in vivo experiment.

At the end of each experiment a dye filled microprobe was used to ionophoretically expel a small quantity of pontamine sky blue dye at several sites. The spinal cord in this area was then removed, fixed in formaldehyde and subsequently sectioned to provide anatomical verification of microprobe placement. An example~~/~~ of one such section is shown in **figure 14**.

FIGURE 14. Histological section of cat spinal cord removed following an in vivo antibody microprobe experiment. Pontamine sky blue was injected in areas of preceding microprobe insertion at a depth of 1.1mm below cord surface (substantia gelatinosa). The location of dye staining in the section has been shaded in the area indicated by the arrow. In this experiment both the site of dye ejection in the substantia gelatinosa and the tract of micropipette insertion have been stained (the latter simply due to dye being forced back up the outside of the micropipette by the pressure produced at the site of ejection). This method was routinely used to verify the placement of microprobes.

FIGURE 14.
VERIFICATION OF IN VIVO
ANTIBODY MICROPROBE PLACEMENT.



2.7 STIMULUS PARAMETERS.

1)Choice of stimulus.

The results presented in this thesis are all derived from experiments in which electrical stimulation at C-fibre strength of a peripheral nerve (the tibial nerve) was employed to elicit tachykinin release in the spinal cord. There were several reasons for this choice of stimulus.

Previous antibody microprobe studies have largely sought to identify the release of particular peptides in the CNS in response to defined, cutaneous stimuli; noxious mechanical, thermal and arthritic challenges have all been employed. The use of a range of stimulus types has two main benefits. Firstly, it increases the likelihood that peptide release will be detected in instances where the exact physiological role of that peptide has not been established. Secondly, it allows some evaluation of what that physiological role is by comparing the efficacy of the different stimulus types in evoking peptide release. The aim of the present studies was different in that we wished to investigate the pharmacological modulation of SP and NKA release in the spinal cord. The stimuli required to evoke release, and the patterns of that release, had already been established for these tachykinins, as discussed in the previous section.

In order to assess whether or not any change in peptide release was truly a pharmacological event, as opposed to the result of physiological variability, we needed a stimulus which would not only

evoke release of tachykinin in the spinal cord, but additionally would not vary with repeated application. Noxious cutaneous stimuli, such as heat and pinch, tend to promote inflammation of the surrounding tissues. This sensitises the peripheral endings of nociceptors (as described in the subsection dealing with tachykinin functions) to subsequent stimulation and thus the central release of neurotransmitter from those nociceptive fibres may increase with time. Nerve stimulation does not normally promote peripheral inflammation and events at the central terminals of nociceptive fibres stimulated electrically should remain constant with repeated stimulation. Electrical stimulation must of course be intermittent to prevent depletion of central release mechanisms.

Nerve stimulation has other advantages. It is much more easily quantified than heat or pinch; the voltage applied to the nerve or the current running through it can be measured very accurately, and monitored throughout the course of the experiment. Electrical stimulation may also evoke release of neuropeptides from 'silent nociceptors' which would not be seen with cutaneous stimuli in the normal animal, and the release of tachykinins from this class of nociceptor may be important in certain inflammatory states (Hope et al, 1990b, Schaible et al, 1990).

In the two studies on pharmacological manipulation of evoked SP release presented here, nerve stimulation was the only stimulus type used. It was chosen for the reasons detailed above. The third study, the earliest work presented, measured the effects of morphine application on the stimulus evoked release of NKA, and employed both noxious mechanical stimulation of the hind paw and nerve stimulation. Because of the persistence of NKA in the spinal cord seen following

its release, it will be evident when comparing the studies that the experimental protocol used was very different from that employed for SP. Because of the nature of this protocol, the stimulus type in the NKA study is of a lesser significance.

2) Protocol for nerve stimulation.

Electrical stimulation of the ipsilateral tibial nerve was employed as a stimulus in all studies reported here. Prior to administration of the neuromuscular blocker, gallamine, the threshold for tibial nerve stimulation was measured. This was taken to be the lowest applied voltage which caused visible twitching of the toe muscles in the hind paw. Experimental stimulation was at 20V, which in all cases exceeded the stimulation threshold by greater than 100 times. Stimuli were delivered as 0.3-0.5ms square wave pulses (singly or in groups of three at 330Hz), at 10-30Hz. These ranges apply to all experiments, but in each individual experiment stimulus parameters were held constant. Stimulation of the tibial nerve at this strength and frequency will activate both unmyelinated (C) and myelinated (A-delta) fibres.

3) Protocol for noxious mechanical stimulation.

Noxious mechanical pinching of the ipsilateral hind paw was employed as a stimulus only in the study on morphine and NKA release. Five 'crocodile' clips were applied to the pads and interdigital skin of the ipsilateral hind paw in a cycle of 3 minutes on, 2 minutes off for the duration of the stimulus (30 minutes total). This pattern of stimulation and the frequency with which it was employed resulted in no visible inflammation of the hind paw.

2.8 ANALYSIS OF ANTIBODY MICROPROBE AUTORADIOGRAPHS.

The techniques employed in the analysis of microprobe images were based on those described by Hendry et al (1988). A satisfactory x-ray film exposure was considered to be one with unblurred, dark images for all microprobes. In some instances, following examination of the initial x-ray film, it was necessary to expose a second film to the microprobes for a different time from that used for the first exposure. Occasionally, single antibody microprobe images would be blurred due to poor apposition of the microprobe to the film and in this case a minor repositioning of the affected microprobe was made prior to repeated exposure. Examples of x-ray film images are included in **figure 15**. The film selected for analysis was then labelled with indelible marker pen, each image being identified by the number assigned to that microprobe prior to its use in vivo. The film was cut up into small sections, each section containing one or two complete microprobe images. Surface dust and grease was removed from each piece of x-ray film by wiping with lens tissue.

Microprobe images were analysed with a computerised image analysis system utilising an Image Technology PC Vision frame grabber board operating in a Data Control Systems 286e(AT) computer. Single microprobe x-ray film images were placed on a standard microscope stage in a light proof box, and illuminated from beneath through a narrow slit only just larger than the microprobe image itself. The film was scanned with a Panasonic charge coupled device camera, producing a 512 x 512 pixel 'map' of the microprobe x-ray film image. The amount of light reaching the camera was kept constant from one x-ray film image to the next by means of varying the current passing to

the light source. The magnification of the system used in these studies was such that the scanned image corresponded to only the last 5mm of the microprobe tip. The system is illustrated in **figure 15**.

Following subtraction of background values, calculated by scanning blank areas of the x-ray film, the scanned images were individually integrated by the computer with respect to darkness for each vertical column of pixels. This resulted in a plot of greyness of the image vs distance along the length of the probe tip with a resolution of 10 μ m between integrals. The principle was illustrated in **figure 6A**. The integrals for each scanned microprobe image were stored on hard disk together with 35 coded values representing various details corresponding to the experimental parameters pertaining to that image. These coded values are outlined in **table 5**. Despite all the checks of microprobe quality performed in siloxane polymer coating and antibody binding procedures, or during in vitro studies, a small proportion of microprobe x-ray film images were found to be unsuitable for further analysis and were discarded at this stage. Such images most often showed focal intensities of radioligand binding which could not be attributed to any physiological event. Subsequently a data sorting program was used to group together microprobe images fulfilling stated experimental criteria. Mean image analyses were then produced for each group, (as illustrated in **figure 6B**), representing mean grey scale value for the group at each 10 μ m point along microprobe length.

For further mathematical analysis of microprobe images, the integral mean grey scale values were pooled into groups of 3, reducing the spatial resolution of the technique to 30 μ m. Thus standard errors of means could be produced for each group of three

points in the mean grey scale plots, and pairs of mean image analyses, representing different experimental manipulations, could be compared statistically.

FIGURE 15. Microprobe image analysis system. (A) Individual x-ray film images of the type shown are placed on a microscope stage, with a sub-stage lighting source, placed in a blacked out cabinet, and scanned by a Panasonic charge-coupled device camera. (B) A direct image of the x-ray film (the last 5mm of the tip) is displayed on a monitor to allow placement and film quality to be assessed. (C) The 512x512 pixel scan is relayed to an Image Technology PC Vision frame grabber board and converted into a data file. (D) A Data Control Systems 286e(AT) computer not only runs the image capture programme, but also subtracts background (blank film) values from each image data file and integrates columns of the 512x512 pixel image into a plot of 'greyness' at 10 μ m intervals along the length of the autoradiograph. The computer stores these grey scale plots, and by running other programmes can be used to search through files of individual images and produce plots of mean grey scale values for groups of microprobes meeting stated experimental criteria. (E) These final images can then be printed out in a form similar to that illustrated throughout sections 3, 4 and 5.

FIGURE 15.
IMAGE ANALYSIS OF ANTIBODY MICROPROBE AUTORADIOGRAPHS.

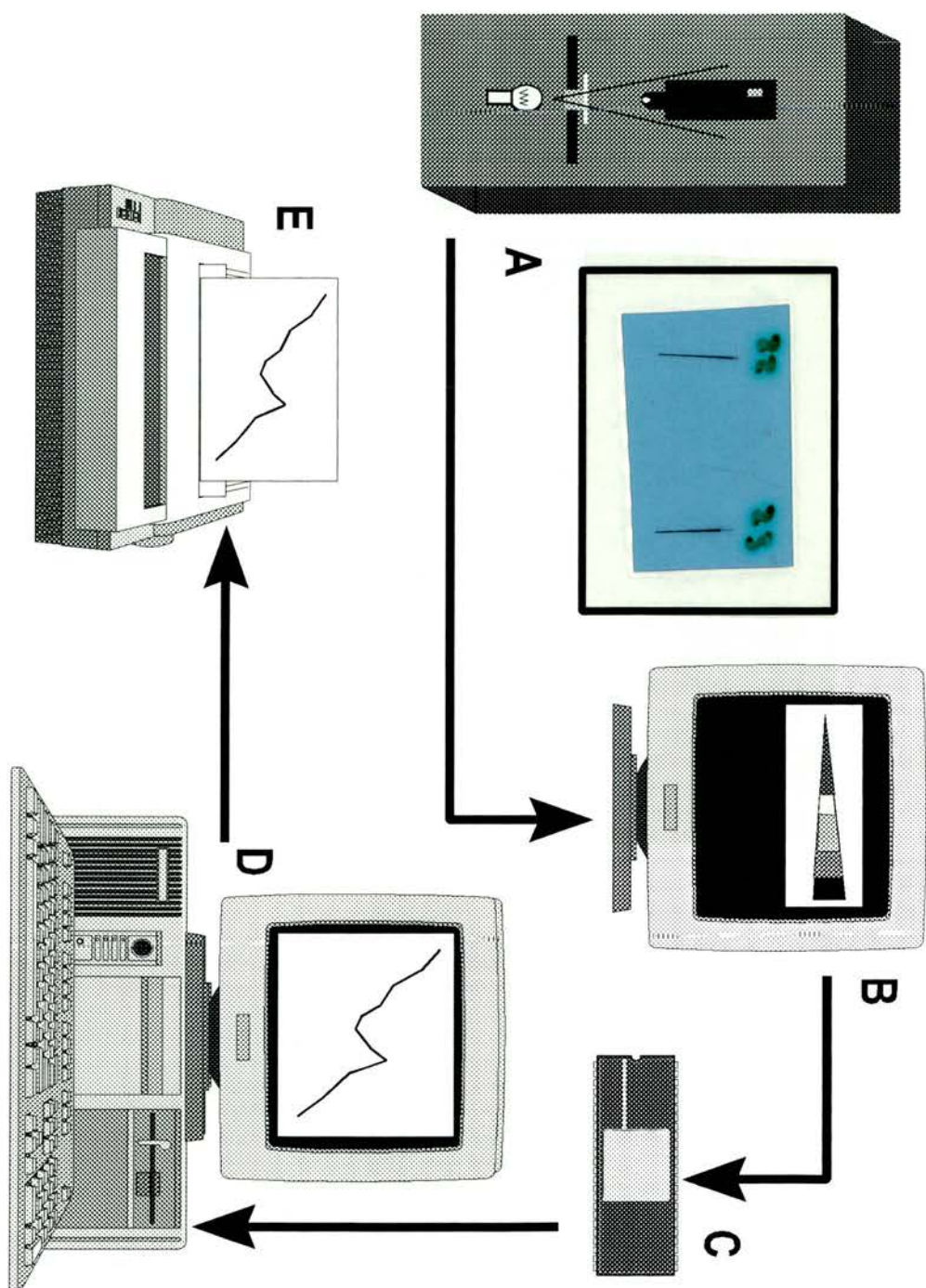


TABLE 5. Examples of coding values applied to individual antibody microprobes. These values allow the subsequent sorting of microprobe images into groups conforming to stated experimental criteria. Some factors (code numbers 1-15) apply to all microprobes in each experiment, whereas others (code numbers 16-35) vary for each individual microprobe. The actual variables for each code are too extensive to list here, but an example (Code 33, drug type and dose) is illustrated.

TABLE 5.
CODED FACTORS FOR INDIVIDUAL MICROPROBES.

<u>Code no.</u>	<u>Description</u>	<u>Code no.</u>	<u>Description</u>
<u>EXPERIMENT VALUES</u>		<u>INDIVIDUAL PROBE VALUES</u>	
1	Record id.number	16	Stimulus type
2	Experiment date (day/month/year)	17	Stimulus parameters
3	Experiment number	18	Individual probe number (in experiment)
4	X-ray film cassette type	19	Time in cord
5	X-ray film type	20	Depth of insertion into cord
6	Hours exposure of film	21	Estimated depth in cord
7	Microprobe glass type	22	Estimated depth in substantia gelatinosa
8	Species (rat/cat)	23	Estimated depth in lamina V
9	Anaesthetic protocol	24	Tracer ligand
10	+/- spinalised	25	Type of wash after in vivo use
11	+/- paralysed (gallamine)	26	Incubation time in radiolabelled ligand
		27	Film faults
12	Antibody used (type and manufacturer)	28	Total prior noxious stimulus for animal
13	Antibody dilution	29	Total prior noxious stimulus for patch
14	Concentration of peptide for in vitro test	30	Age of patch
15	Method of spinal cord irrigation	31	Probe order in patch (1st, 2nd etc)
		32	Antibody type on paired probe
		33	Drug type and dose
		34	Time since first dose of active drug
		35	Extra comments (mark for deletion etc.)

<u>EXAMPLE</u>	<u>VALUES</u>
Code number 33, Drug type and dose. From morphine study (section 3)	
Morphine 0-5mg/kg i.v.	10
Morphine 5.1-10 mg/kg i.v.	11
Morphine >10mg/kg i.v.	12
Naloxone (after morphine)	13
0-0.5mg/kg i.v.	14
Naloxone (after morphine)	15
0.51-1mg/kg i.v.	16
Nalox. only 0-0.5mg/kg i.v.	17
Nalox. only 0.51-1mg/kg i.v.	18
Nalox. only >1.0mg/kg i.v.	19

<p style="text-align: center;"><u>SECTION 3.</u></p> <p style="text-align: center;"><u>THE EFFECTS OF MORPHINE</u></p> <p style="text-align: center;"><u>ON NEUROKININ A RELEASE.</u></p>

The results presented in this section have previously been published as:

"Analgesic doses of morphine do not reduce noxious stimulus-evoked release of immunoreactive neurokinins in the dorsal horn of the spinal cat." C.W.Lang, A.W.Duggan & P.J.Hope.

British Journal of Pharmacology (1991) volume 103, pp 1871-1876.

These experiments were the first series during the course of my studies in which I was the principal investigator.

3.1 INTRODUCTION.

Opiate drugs are used in both veterinary and, more widely, in human medicine, where they are of particular use as analgesics, and are notably effective when administered epidurally (see Benedetti et al, 1990 for review of current therapeutic techniques). Despite this clinically proven efficacy in alleviating the transmission and perception of pain, the mechanisms by which opiate drugs bring about such effects have not yet been fully established.

Substance P (SP) and neurokinin A (NKA), two members of the tachykinin family of neuropeptides, extensively coexist in the small diameter dorsal root ganglion cells of the rat (Dalsgaard et al, 1985, Dalsgaard, 1988). Both of these peptides have been proposed as being important in the transmission of information from nociceptive afferents to spinal or brainstem neurones (Otsuka and Yanagisawa, 1988, Fleetwood-Walker et al, 1990). Studies of the release of these compounds in the spinal cord of the cat with antibody microprobes have shown that both SP and NKA are released in response to a similar range of peripheral noxious stimuli (Duggan et al, 1988b, 1990, Hope et al, 1990b, Schaible et al, 1990), including noxious thermal and mechanical challenge. The findings of these studies suggested that higher grade noxious stimuli which produced actual tissue damage, as opposed to non-damaging noxious stimuli, might be more effective in releasing immunoreactive (ir) SP. irNKA detection on the other hand was not increased by tissue damage. One important difference between the release patterns of the two peptides was, however, in their persistence in the spinal cord following noxious stimulus-evoked release. Relative to SP, NKA spread widely in the spinal cord

following its release, and could be detected for a considerable period of time (up to one hour) in the absence of continued stimulation. This led to the hypothesis that NKA may not function as a classical neurotransmitter, but might instead have a long term modulatory role on spinal cord systems.

Opioid binding sites (unclassified: Atweh and Kuhar, 1977, rat, Pert et al, 1975, rat, Lamotte et al, 1976, primate, mu: Morris and Herz, 1987, rat, Gouarderes et al, 1985, rat and guinea-pig, delta: Dashwood et al, 1986, rat, Traynor et al, 1990, rat, mouse, hamster, mouse, kappa: Allerton et al, 1989, rat, Hunter et al, 1989, dog) and endogenous opioid peptides such as dynorphin and enkephalins (Botticelli et al, 1981, rat and rabbit, Lamotte et al, 1991, primate, Senba et al, 1989, rat) have been demonstrated in the spinal cord, and are largely associated with the superficial laminae of the dorsal horn. Systemic morphine administration in spinal cats (Einsphar and Piercey, 1980, Le Bars et al, 1976a,b) spinal rats (Jurna and Heinz, 1979) and paraplegic man (Willer and Bussel, 1979) reduces the responses of neurones in the superficial and deep laminae of the dorsal horn to noxious thermal, noxious mechanical and C/A delta fibre stimulation. The effects of electrical stimulation of larger diameter nerve fibres was unaffected as were spinal neurones identified as having a purely non-noxious input, suggesting that the inhibitory effects of morphine might be specific to nociceptive afferent pathways. Ionophoretic application of morphine (Duggan et al, 1977) and the μ opioid agonist [D-Ala² Me-Phe⁴, Gly-ol⁵] enkephalin (DAMGO) (Fleetwood-Walker et al, 1988) in the region of the substantia gelatinosa (SG) of the cat selectively suppressed the transmission of impulses from nociceptive afferents to deeper

neurones of the dorsal horn. The SG is the major site of termination of unmyelinated cutaneous afferent fibres (Light and Perl, 1977). Since administration of naloxone into the SG completely blocked the effects of analgesic doses of systemically administered morphine on deep neurones of the dorsal horn of the cat (Johnson and Duggan, 1981), it is likely that opiate receptors in the SG are the major ones mediating the analgesic actions of morphine at the spinal level, whether administered systemically or intrathecally.

A variety of mu and delta opiate receptor agonists, when applied topically, produce inhibition of spinal cord responses to electrically-evoked C-fibre input (Dickenson et al, 1986, 1987, 1988, Sullivan et al, 1989). Results of such studies are valuable in confirming the importance of these receptor subtypes in spinal processing of nociceptive input, but cannot identify which sites within the dorsal horn are involved.

Met⁵-enkephalin (a delta agonist), morphine and metorphamide (a mu and partial kappa agonist) microinjected into the SG all depressed the responses of neurones in laminae IV and V to peripheral noxious thermal and C/A-fibre stimulation, but only met⁵-enkephalin showed this effect when microinjected directly into laminae IV/V (Duggan et al, 1981, Morton et al, 1987, Zhao et al, 1986). These results suggest that both mu and delta opioid receptor-mediated inhibitory mechanisms may exist in the substantia gelatinosa and that, additionally, delta opioid inhibitory mechanisms exist in direct association with neuronal structures in the deeper laminae of the spinal dorsal horn. The effects of [D-Ala², D-Leu⁵]-enkephalin (DADL), a highly selective delta opioid agonist, also support the existence of a delta opioid system mediating the inhibition of spinal

nociceptive afferent transmission (Onofrio and Yaksh, 1983, Tung and Yaksh, 1982, Yaksh, 1983). Kappa opioid agonists such as ethylketocyclazocine, bremazocine and U-50,48811 (a benzeneacetamide derivative), when administered intrathecally in the rat were shown to inhibit behavioural responses to noxious visceral chemical stimuli but not to noxious cutaneous thermal stimuli (Schmauss and Yaksh, 1984). Intrathecal administration of mu agonists in the rat also inhibit the typical grooming/scratching response to intrathecal neurokinin administration (Wilcox, 1988). One recent study has suggested that kappa opioid receptors exert their antinociceptive effects predominantly in the deeper laminae of the dorsal horn (Fleetwood-Walker et al, 1988). The same study failed to detect any antinociceptive effect, in either the SG or in the deeper laminae of the dorsal horn, of delta opioid agonists against noxious cutaneous thermal or mechanical stimulation. This same research group also reported antinociceptive effects of delta ligands and, to a lesser extent of mu ligands, in lamina I of the rat dorsal horn (Fleetwood-Walker et al, 1988, Hope et al, 1990a).

The functional distribution of opioid receptor types in the spinal dorsal horn is clearly complex and is not yet fully resolved. It seems likely however that with respect to the processing of nociceptive inputs in the rat, delta, mu and kappa receptors are of primary importance in laminae I-II, II-III (the SG) and laminae III-V respectively (Duggan and Fleetwood-Walker, 1993). The relative contributions of each receptor type may, however, change with different pain states. For example, Stanfa et al (1992) reported that during carrageenan-induced peripheral inflammation in the rat the potencies of intrathecally applied mu, delta and kappa opioid

receptor agonists for the depression of spinal cord neuronal activity were all increased. The potency of the mu agonist, morphine, was enhanced to a far greater extent (30 times normal) than the other agonists, suggesting that mu opioid receptor mediated inhibition may be predominantly important in the processing of nociceptive input from inflamed tissues. The role of opioid peptides and related drugs in spinal sensory processing has recently been reviewed (Duggan and Fleetwood-Walker, 1993).

One mechanism by which morphine could produce analgesia is by a presynaptic inhibition of neurotransmitter release from nociceptive primary afferent terminals. An early observation in support of this hypothesis was that the endogenous opioid peptide, enkephalin, inhibited the release of SP from cultured sensory neurones (Mudge et al, 1979). Morphine, in high concentrations, when added to the superfusate of either a slice of upper spinal dorsal horn (Mauborgne et al, 1987b, Pang and Vasko, 1986), or of the spinal cord in vivo (Go and Yaksh, 1987, Hirota et al, 1985, Yaksh et al, 1980) has been shown to reduce stimulus evoked release of irSP. However, systemically administered morphine at analgesic doses has failed to reduce release of irSP evoked by noxious stimulation (Kuraishi et al, 1983, Morton et al, 1990).

With the recognition that a peripheral noxious stimulus results in the central release of a number of neuroactive compounds including amino acids (Kangrga and Randic, 1990) and peptides (Duggan et al, 1988b, 1990), a complete study of opiates and release from primary afferents is clearly a complex task. There is evidence that the arrival of impulses in nociceptive afferents produces not only fast transmission to fibres ascending to the brain, but also long lasting

events such as prolonged facilitation of flexor reflexes (Cervero et al, Cook et al, 1987, Hoheisel and Mense, 1989, Hylden et al, 1989, 1988, Neugebauer and Schaible, 1988, Woolf, 1983, Woolf and Wall, 1986a). It has been proposed that NKA (rather than SP) is more important in these prolonged spinal events as, following release of irNKA, this peptide (or an extended form such as neuropeptide K (NPK)) diffuses widely away from the initial sites of release and also persists in the spinal cord (Hope et al, 1990b,c). Of note is the finding that, in the rat, low doses of morphine suppressed the prolonged facilitation of spinal reflexes produced by nociceptive input, whereas higher doses were required to suppress fast transmission as measured by an unfacilitated nociceptive reflex (Woolf and Wall, 1986b). Such considerations indicate the need for a study of opiates and the central release of NKA. In the present study therefore, the effects of analgesic doses of systemically administered morphine on the noxious stimulus-evoked release of NKA were examined in the spinal cords of barbiturate anaesthetised, spinalised cats.

3.2 ANTIBODY MICROPROBE PREPARATION.

Antibody microprobes were prepared as previously described. A polyclonal antiserum, raised against the carboxyl terminus of NKA was used (Peninsula). Information supplied by the manufacturer indicated that this antiserum showed 100% cross-reactivity for the N-terminal extended form of NKA, NPK, and 70-80% cross-reactivity with neurokinin B (NKB), but negligible cross reactivity with SP. This was verified by in vitro tests as described earlier.

3.3 ANIMAL PREPARATION/MICROPROBE ANALYSIS.

Experiments were performed on 8 cats, anaesthetised and subsequently prepared as described under the section on general methodology. All microprobes were inserted 3mm into the dorsal spinal cord, which in this species places their tips in the upper ventral horn. All microprobes remained in the spinal cord for 30 minutes. The analysis of antibody microprobes subsequent to their use in vivo and in vitro also followed the methods outlined earlier.

3.4 STIMULUS PARAMETERS.

Peripheral noxious stimulation throughout a 30 minute period of antibody microprobe insertion was provided either 1)by applying 'crocodile' clips to the pads and interdigital skin of the ipsilateral hind paw in a cycle of 3 minutes on, 2 minutes off or 2)by electrical stimulation of the ipsilateral tibial nerve (groups of three 0.5ms pulses at 330Hz, repeated at 10Hz, amplitude 20V).

3.5 DRUG ADMINISTRATION AND EXPERIMENTAL REGIMES.

The drugs used were administered via an intravenous catheter placed in the right cephalic vein. Morphine was administered as a 10mg ml⁻¹ solution in physiological saline, naloxone as 1mg ml⁻¹ in physiological saline.

As previously demonstrated (Duggan et al, 1990, Hope et al, 1990c), the elevation of irNKA levels in the dorsal spinal cord following a limited peripheral noxious stimulus, persists for up to 1 hour. Such persistence creates difficulties when testing a compound which may block release, as a series of pre-drug measurements of stimulus-evoked release may result in a build up of irNKA in the dorsal horn. In this situation any subsequent reduction in neurokinin release brought about by drug administration may not be detectable. For this reason, these experiments examined the basal levels of irNKA present in the dorsal horn in the absence of any prior noxious stimulation and then administered morphine before measuring the stimulus evoked release of these compounds. Several groups have shown that, in the spinal cat, doses of morphine ranging from 0.3 to 4.0mg kg⁻¹ iv. reduced the synaptic activation of dorsal horn neurones by peripheral noxious stimuli (Duggan et al, 1980, Johnson and Duggan, 1981, Le Bars et al, 1976a,b). The present experiments used morphine at doses just above the upper limit of this range (5.0mg kg⁻¹iv.) as, with the protocol used, a complete inhibition of noxious stimulus-evoked irNKA release would be much more readily detected than a partial reduction. A more significant test of the effect of morphine on irNKA release came from the effects of subsequent naloxone administration. If stimulus-evoked release of

irNKA was increased when naloxone is administered after morphine, this would indicate that morphine had reduced, even if only partially, the release of these compounds.

The above protocol is not optimal if morphine increases the release of irNKA. A series of experiments were therefore included in which the effects of both morphine and/or naloxone on basal levels of irNKA were evaluated and comparisons were also made with earlier studies (Hope et al, 1990b,c) on the patterns of stimulus-evoked irNKA release in the absence of pharmacological manipulation.

3.6 RESULTS.

A total of 97 microprobes coated with antibodies to NKA were inserted into the dorsal spinal cord. Additionally, 64 microprobes were used for concurrent in vitro tests performed as previously described. These in vitro tests consistently demonstrated suppression of the binding of ^{125}I -NKA by greater than 50% following prior incubation in a solution of 10^{-7} mol l^{-1} unlabelled NKA.

35 antibody microprobes were inserted 3mm into the spinal cord and left in situ for 30 minutes without stimulation of either hind paw or the concurrent administration of any drug. These 'control' microprobes formed the basis for assessing possible effects of morphine or naloxone on the basal levels of irNKA. The remaining 62 probes were inserted into the spinal cord in the presence of peripheral stimuli and/or drug administration as detailed in the following pages.

3.6.1 EFFECTS OF MORPHINE AND NALOXONE ON BASAL LEVELS OF IMMUNOREACTIVE NEUROKININS.

In 4 experiments morphine was the first drug administered, and in the remaining experiments naloxone was administered first.

7 probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimulation, and within 1 minute of morphine administration (5mg kg^{-1} iv.). In the case of these microprobes, this dose of morphine represented the first drug administration. The mean image analysis of these microprobes is illustrated in the upper trace ('morphine') of **figure 16**. When this group is compared with the mean image analysis of microprobes inserted into the spinal cord in the absence of any prior stimulation or drug application and in the same experiments (lower trace **figure 16**, 'controls', $n=11$), no significant differences are present, as illustrated in **figure 16** in the plot of the t-statistics derived from the differences between these two mean image analyses.

13 microprobes were inserted into the dorsal spinal cord in the absence of prior or concurrent noxious stimulation and within 1 minute of naloxone administration (0.5mg kg^{-1} iv.). In the case of these microprobes, there had been no prior morphine administration. The mean image analyses of this group of microprobes ('naloxone') and of their corresponding control group ('controls', $n=23$) are illustrated in **figure 17** together with a plot of the t-statistics derived from their differences. This plot of t-values shows that no significant differences exist between the two groups.

An additional 10 microprobes were inserted into the spinal cord in the absence of any prior or concurrent noxious stimulation and within 1 minute of morphine application (5mg kg^{-1} iv.), but in this case morphine administration immediately followed the application of naloxone (0.5mg kg^{-1} iv.). Again, when compared with the relevant control group, no statistically significant differences were found (figure 18).

Summary. Neither morphine (5mg kg^{-1} iv.) nor naloxone (0.5mg kg^{-1} iv.), administered in the absence of peripheral noxious stimulation, produced significant changes in the detectable basal levels of irNKA in the dorsal spinal cord. A combination of the two drugs at the same dosages also failed to significantly alter basal levels of irNKA in the dorsal horn.

3.6.2 EFFECT OF MORPHINE ON STIMULUS-EVOKED IMMUNOREACTIVE NEUROKININ RELEASE.

17 microprobes were inserted into the dorsal spinal cord within 90 minutes of morphine administration (5mg kg^{-1} iv.) and with concurrent noxious stimulation of the ipsilateral hind paw or tibial nerve. Noxious pinch and electrical stimulation of unmyelinated primary afferent fibres have previously been shown to be adequate stimuli for releasing irNKA in the dorsal spinal cord (Duggan et al, 1990, Hope et al, 1990c). In the series of experiments illustrated here, following morphine administration, noxious stimulus-evoked irNKA release was observed in a zone extending from the spinal cord

surface to approximately 1.3mm into the dorsal grey matter. This is illustrated in **figure 19**. Although these increases in the levels of irNKA in the dorsal horn produced by peripheral noxious stimulation appear similar to those produced by comparable stimuli in other studies in which drugs were not administered (Duggan et al, 1990, Hope et al, 1990b,c) a small inhibition of release by morphine could only be assessed by the subsequent administration of naloxone.

11 microprobes were inserted into the dorsal spinal cord within 1 minute of naloxone administration (0.5mg kg^{-1} iv.) given after morphine application and with concurrent peripheral noxious stimulation. These microprobes were inserted immediately following those illustrated in **figure 19**, immediately after the period of peripheral stimulation following morphine administration. **Figure 20** compares the mean image analyses of the two groups and shows that naloxone administration had no significant effect on the levels of noxious stimulus-evoked irNKA detection in the spinal dorsal horn seen in the presence of systemic morphine. This lack of change following naloxone administration suggests that prior morphine application had in no way altered noxious stimulus-evoked irNKA release.

Summary. Morphine application (5mg kg^{-1} iv.) did not result in a complete inhibition of noxious stimulus-evoked irNKA release. Furthermore, the pattern of irNKA detection seen following subsequent naloxone administration (0.5mg kg^{-1} iv.) suggests that morphine had not produced a partial reduction in stimulus-evoked irNKA release.

FIGURE 16. The effects of systemic morphine administration on resting levels of immunoreactive neurokinin A in the dorsal horn. 'Controls', the mean image analysis of microprobes (n=11) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. 'Morphine', the mean image analysis of microprobes (n=7) inserted into the dorsal spinal cord immediately following morphine (5mg kg^{-1} , i.v.) administration and in the absence of prior or concurrent peripheral stimulation. The analysis was performed with a resolution of $10\mu\text{m}$ and with each group the continuous line has joined these points. The + standard error of the mean (SEM) at each point is plotted for the 'morphine' group and the -SEM at each point is plotted for the 'control' group. The t-values ($2t = P < 0.05$) derived from the differences between the means of the two groups are also shown.

FIGURE 16.
THE EFFECT OF MORPHINE ADMINISTRATION ON BASAL LEVELS OF NEUROKININ A.

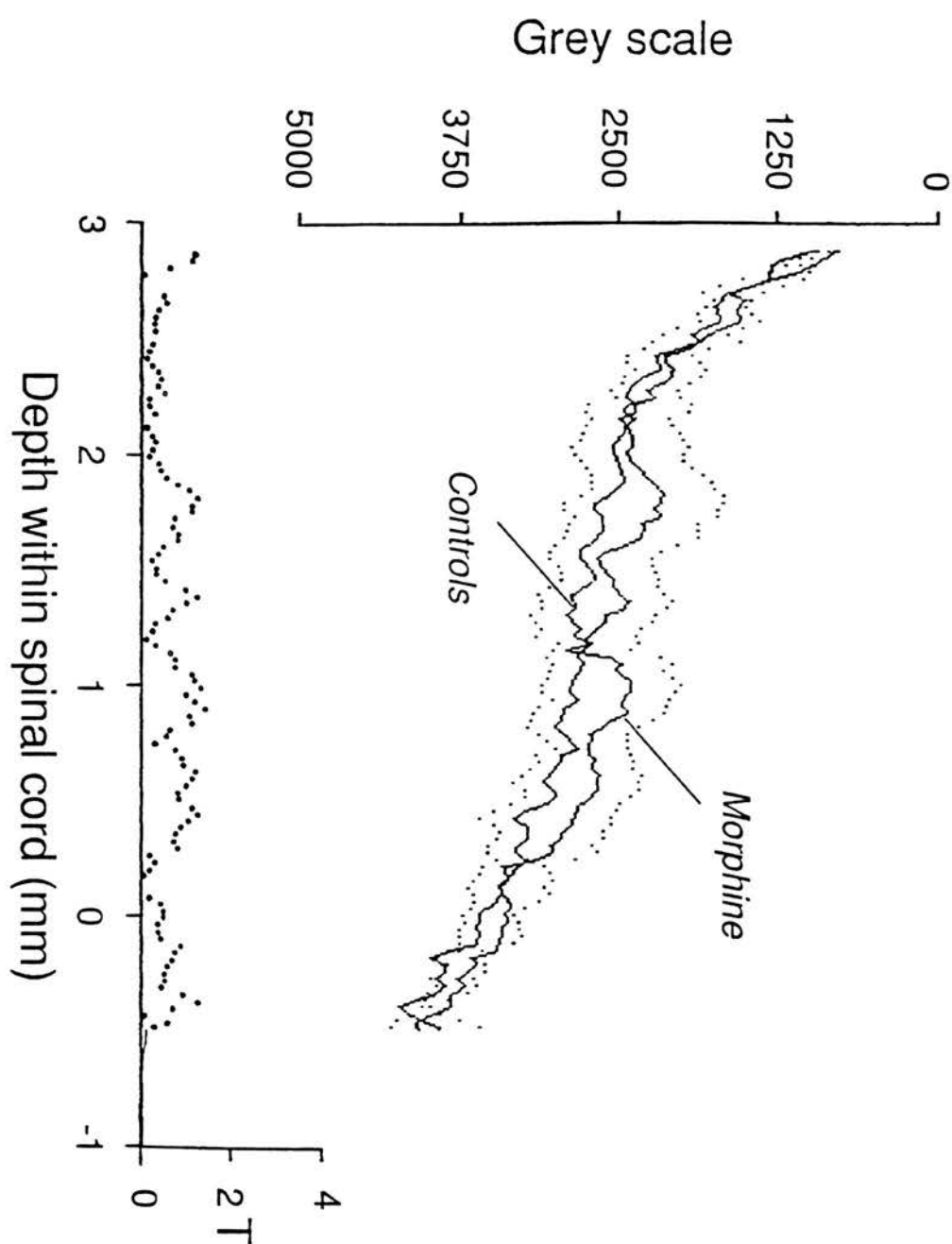


FIGURE 17. The effects of systemic naloxone administration on resting levels of immunoreactive neurokinin A in the dorsal horn. 'Controls', the mean image analysis of microprobes (n=23) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. 'Naloxone', the mean image analysis of microprobes (n=13) inserted into the dorsal spinal cord immediately following naloxone (0.5mg kg^{-1} , i.v.) administration and in the absence of prior or concurrent peripheral stimulation. Standard errors of means for the two groups and t values ($2t=P<0.05$) derived from the differences of the means are shown as in figure 16.

FIGURE 17. THE EFFECT OF NALOXONE ADMINISTRATION ON BASAL LEVELS OF NEUROKININ A.

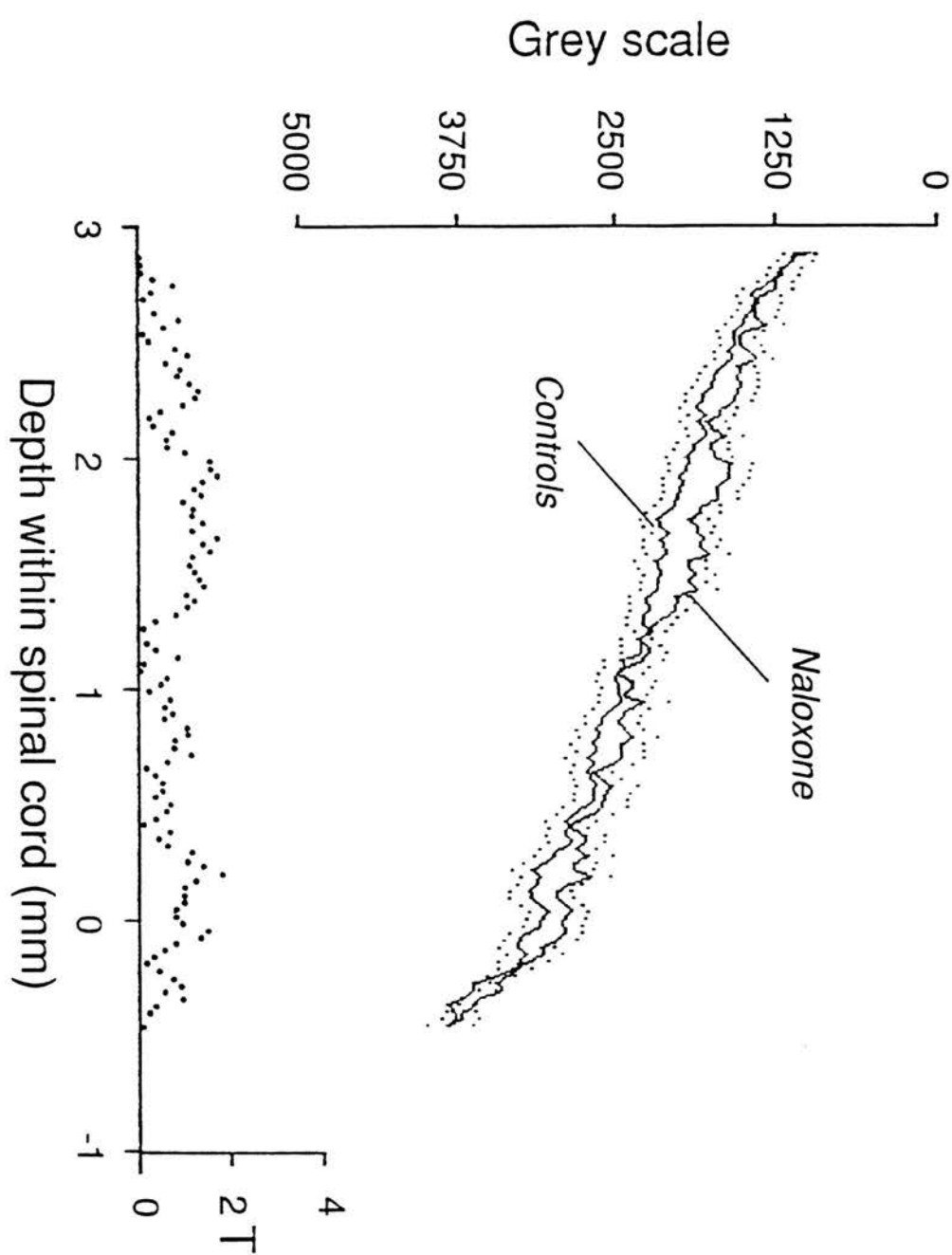


FIGURE 18. The effect of systemic morphine following systemic naloxone on resting levels of immunoreactive neurokinin A in the dorsal horn. 'Controls', the mean image analysis of microprobes (n=11) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. 'Morphine following naloxone', the mean image analysis of microprobes (n=10) inserted into the dorsal spinal cord following morphine (5mg kg⁻¹, i.v.) which immediately followed naloxone (0.5mg kg⁻¹, i.v.) administration and in the absence of prior or concurrent peripheral stimulation. Standard errors of means for the two groups and t values (2t=P<0.05) derived from the differences of the means are shown as in figure 16.

FIGURE 18.
THE EFFECT OF MORPHINE ADMINISTRATION ON BASAL LEVELS OF NEUROKININ A
FOLLOWING NALOXONE.

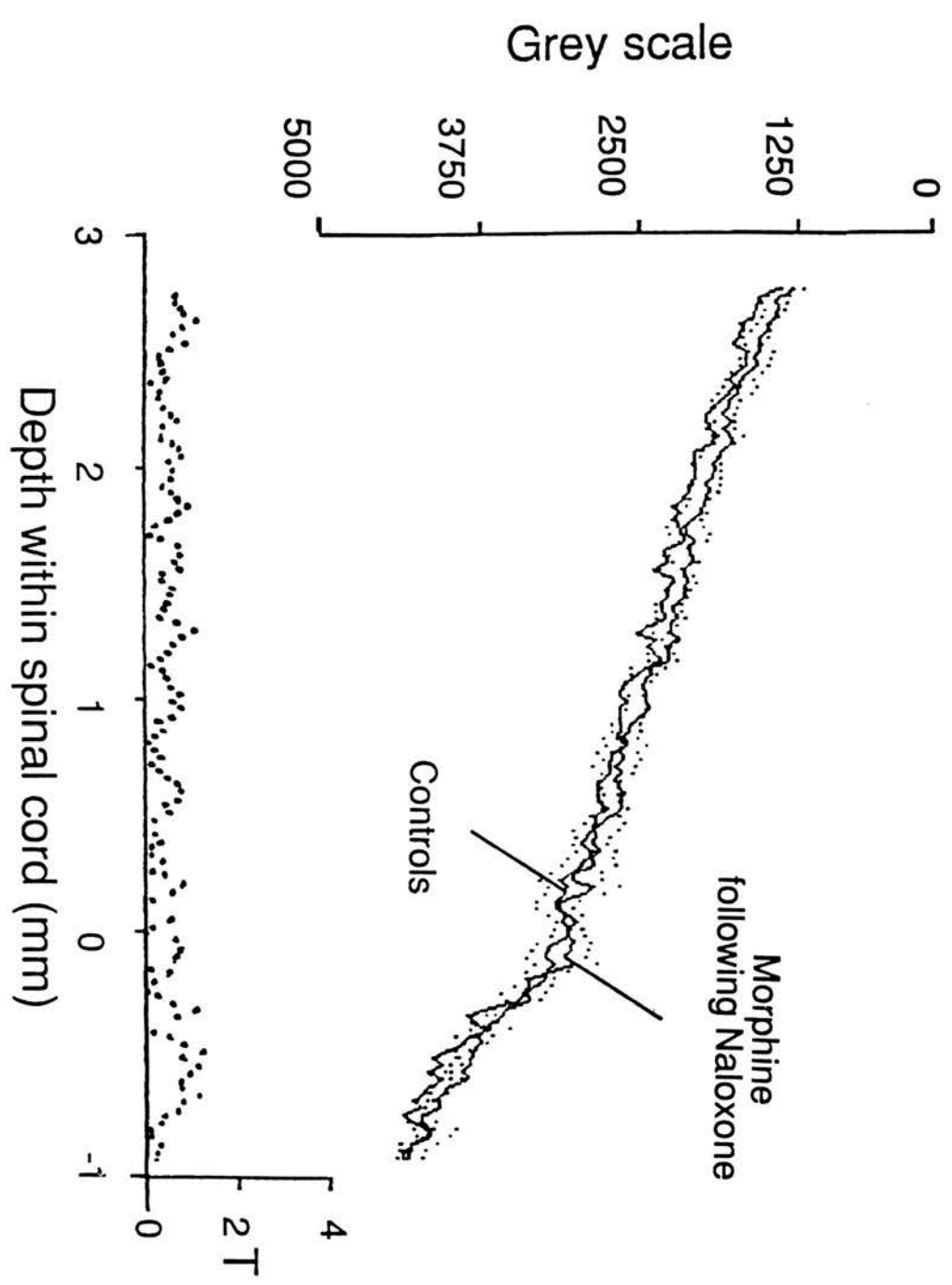


FIGURE 19. The effects of noxious stimulation of the ipsilateral hind paw, following systemic morphine administration on immunoreactive neurokinin A levels in the dorsal horn. 'Controls', the mean image analysis of microprobes (n=11) inserted into the dorsal spinal cord with no prior or concurrent peripheral stimulation. 'Morphine (stim.)', the mean image analysis of microprobes (n=17) inserted into the dorsal spinal cord following morphine (5mg kg^{-1} , i.v.) administration and during noxious stimulation of the ipsilateral hind paw. Standard errors of means for the two groups and t values ($2t=P<0.05$) derived from the differences of the means are shown as in figure 16.

FIGURE 19.
THE EFFECT OF MORPHINE ADMINISTRATION ON STIMULUS-EVOKED LEVELS OF NEUROKININ A.

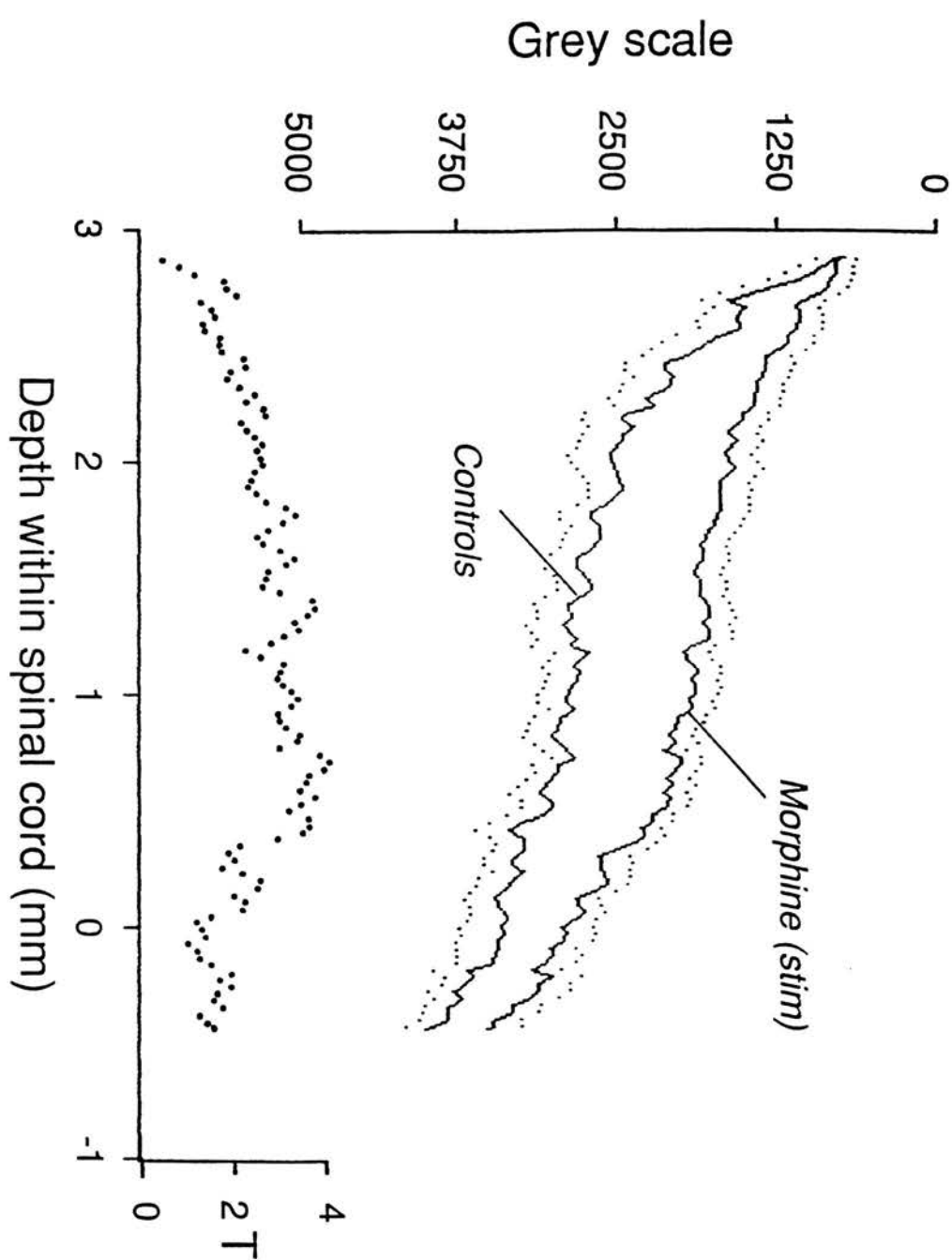
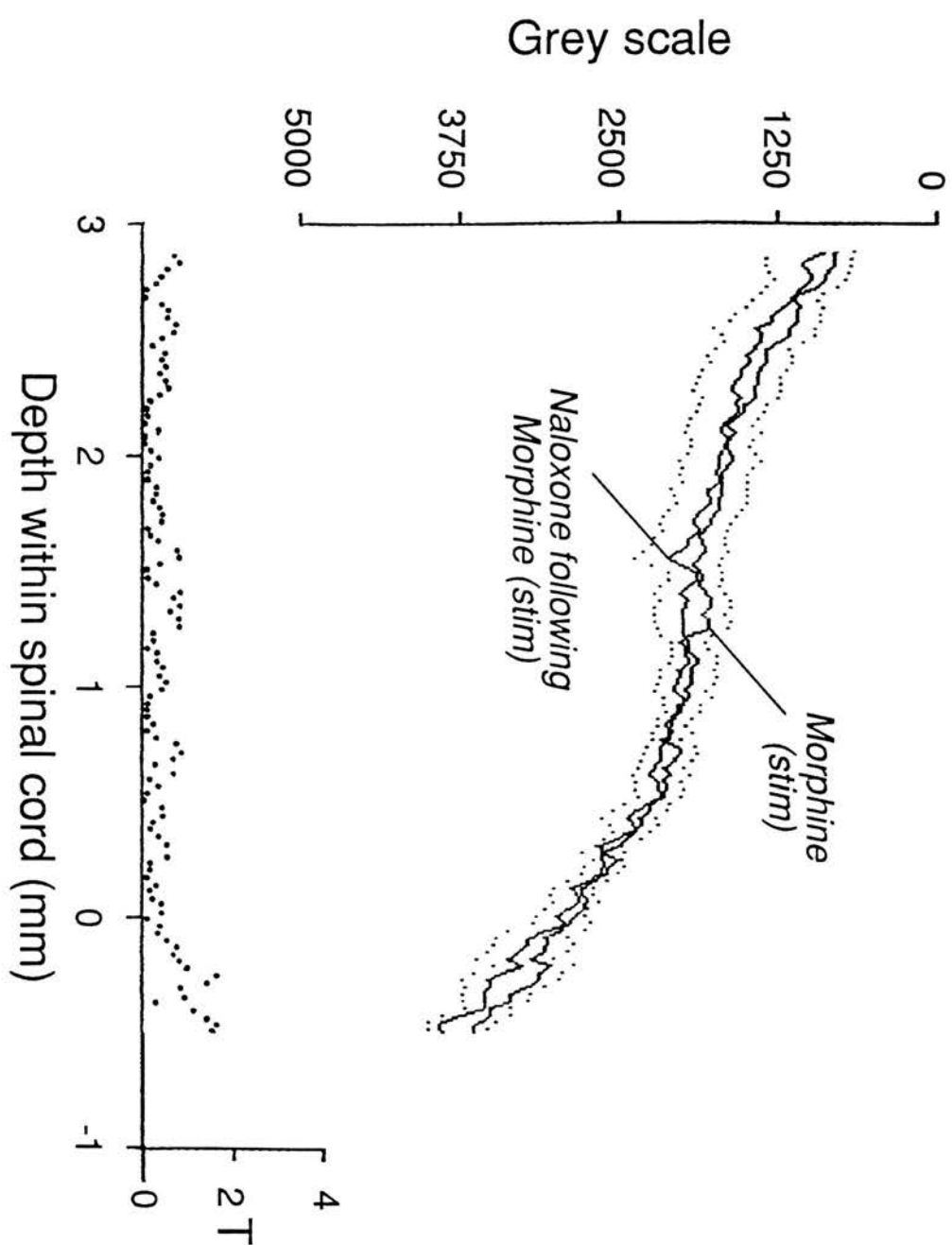


FIGURE 20. Effect of naloxone following morphine on noxious stimulus-evoked immunoreactive neurokinin A release. 'Morphine (stim.)', the mean image analysis of microprobes (n=17) as described in figure 19. 'Naloxone following morphine (stim.)', the image analysis of microprobes (n=10) inserted into the dorsal spinal cord immediately following those illustrated in the 'Morphine (stim.)' group, and after naloxone (0.5mg kg^{-1} , i.v.) administration during noxious stimulation of the ipsilateral hind paw. Standard errors of means for the two groups and t values ($2t=P<0.05$) derived from the differences of the means are shown as in figure 16.

FIGURE 20.
THE EFFECT OF NALOXONE ADMINISTRATION ON STIMULUS-EVOKED LEVELS OF NEUROKININ A
FOLLOWING MORPHINE.



3.7 DISCUSSION.

When the results of the present experiments are considered with those of Kuraishi et al (1983) and Morton et al (1990), there is little evidence that analgesic doses of opiates administered systemically reduce tachykinin release (either SP or NKA) from the central terminals of nociceptors. These results are, however, at variance with those of studies examining the effects of topically applied opiates on SP release, and these differences merit further discussion.

Other studies on morphine and SP release.

Inhibition of SP release by opiates was first shown by Jessell and Iversen (1977) in a slice preparation of rat trigeminal nucleus. The stimulus used in this study was, however, 47mM K⁺ in the perfusate. A similar effect on high K⁺ induced SP release has been demonstrated with slices of rat spinal cord in vitro (Lembeck and Donnerer, 1985, Mauborgne et al, 1987b, Pang and Vasko, 1986). The main problem with such studies is in the stimulus employed. Elevation of K⁺ levels in the slice perfusate will depolarise virtually all neuronal structures. SP is closely associated with primary afferent terminals of nociceptive origin in the superficial laminae of the dorsal horn, but as has previously been discussed a significant proportion of neuronal elements in the spinal cord showing immunoreactivity for SP represent the terminals of either descending fibres of supraspinal origin or of intrinsic spinal neurones. Hence the source of SP in the above studies is uncertain. Furthermore, the concentration of morphine employed was high, 10⁻⁴-10⁻⁵M.

Using the push-pull cannula technique on rabbit spinal cord in vivo, Hirota et al (1985) demonstrated a reduction in the release of SP following noxious cutaneous stimulation with 10^{-5}M morphine in the perfusate. Further support for the proposal that opiates impair SP release from the central terminals of nociceptors came from the experiments of Go and Yaksh (1987) and Yaksh et al (1980) in which 10^{-4}M morphine added to a superfusate of cat spinal cord in vivo reduced the release of SP into the perfusate following electrical stimulation of unmyelinated primary afferent fibres.

The roles of mu, delta and kappa receptors in opiate spinal analgesia.

The contributions of the different opiate receptor types to inhibition of stimulus-evoked SP release have also been studied. The group of Mauborgne et al (1987b) found that while the mu receptor agonist [D-Ala²,N-Me-Phe⁴, -Gly-ol]-enkephalin (DAMGO) (10^{-5}M) increased both high K^+ and capsaicin induced release of SP from rat dorsal horn slices, the delta-receptor agonist [D-Thr²]-Leu-enkephalin-Thr (DTLET) (3.10^{-6}M) reduced SP release in the same preparation. Aimone and Yaksh (1989) found that both mu (morphine) and delta (DAGO, or [D-Pen², -Pen⁵]-enkephalin (DPLPE)) opioid receptor agonists, all at 10^{-6} - 10^{-5}M , were effective in inhibiting capsaicin-evoked SP release from rat spinal cord in vivo. DTLET and dermenkephalin, another delta agonist, have also been shown to inhibit the basal release of SP into an intrathecal perfusate of rat spinal cord in vivo (Collin et al, 1991). The same group demonstrated an enhancing effect of the delta antagonist naltrindole on these basal SP levels, suggesting the existence of a tonic delta opioid

receptor mediated inhibition. A role for delta receptors in mediating the inhibitory effects of opiates on neuronal activity in the spinal cord has clearly been identified in many studies (Dickenson et al, 1986,1987,1988, Onofrio and Yaksh, 1983, Sullivan et al, 1989, Tung and Yaksh, 1982, Yaksh, 1983). Kappa receptor agonists such as U-50,488H ($0.5 - 50.10^{-6}M$) have been shown to be without effect on the release of SP from the spinal cord in a variety of in vitro and in vivo preparations (Aimone and Yaksh, 1989, Hamon et al, 1988, Pohl et al, 1989).

The effects of drug dose.

It might be noted that the concentrations of drugs used in all of the above studies were high and that no group has found that systemic analgesic doses of morphine reduce SP release in the spinal cords of spinalised animals. Thus, Kuraishi et al (1983) using push-pull cannulae in the dorsal horn of the spinal rabbit observed that although systemic morphine at $10mg\ kg^{-1}$ iv reduced irSP release in response to peripheral noxious mechanical stimulation, a lower dose of $1mg\ kg^{-1}$ iv was ineffective in producing such a change. Morton et al (1990), using antibody microprobes, failed to observe any effect of morphine in two dose ranges ($1-6mg\ kg^{-1}$ and $10-20mg\ kg^{-1}$ iv.) on irSP release in the dorsal horn of the spinal cat following noxious mechanical or thermal cutaneous stimulation.

Since studies which have observed effects on tachykinin release have used relatively high concentrations of opiates, is the same true of experiments which have administered opiates microionophoretically in the substantia gelatinosa (SG) and hence may have over-emphasised the importance of events near the first central synapse of

nociceptors? Although tissue concentrations in microionophoretic experiments are unknown, the finding that naloxone administered in the SG decreased the effects of systemic morphine (4mg kg^{-1}) on deeper neurones of the spinal cord (Johnson and Duggan, 1981) does support the importance of opiate receptors in the SG in mediating the action of systemic morphine on spinal neurones. This still does not identify a direct or indirect presynaptic action of opiates on the central terminals of nociceptive afferent fibres, and the conflicting evidence for and against such an effect comes principally from, in some cases, more refined electrophysiological studies.

Cellular mechanisms for morphine analgesia, the evidence for pre- and postsynaptic effects.

Support for a postsynaptic effect of opioids on intrinsic neurones of the SG came from Yoshimura and North (1983) who observed a hyperpolarisation (possibly mediated by an increased K^+ conductance) of such cells in a slice preparation, and Sastry and Goh (1983) who found many to be excited in vivo by ionophoretically administered morphine. These inconsistencies have not, however, been explained.

Morphine has been shown to have a stimulatory effect on Na^+/K^+ -ATPase activity in homogenates of rat cerebral cortical synaptic membrane or synaptosomal membrane (Nishikawa et al, 1990, Nishikawa and Shimuzu, 1990). This effect would support both presynaptic and postsynaptic inhibitory mechanisms, but would be totally nonselective within the neuronal population of the dorsal horn. Other studies have associated the action of morphine with a specific receptor-linked G

protein mediating K^+ membrane conductance (Shen and Crain, 1990), ~~allowing for a tissue selective postsynaptic effect.~~

When gamma-aminobutyric acid (GABA) acts presynaptically on the central terminals of large diameter muscle afferents, the resulting depolarisation is associated with reduced transmitter release and an increased excitability when tested with an adjacent stimulating microelectrode (Curtis et al, 1980). Administering opioid peptides near the central terminals of nociceptors in the SG has, however, been shown to result in decreased electrical excitability (Carstens et al, 1987, Sastry, 1980), a result not consistent with the well established mechanism of GABA mediated presynaptic inhibition. A different mechanism of reducing transmitter release from central terminals might be if calcium entry with each invading impulse were to be reduced through a shortening of action potential duration. Such a shortening has been observed with micromolar concentrations of opioids acting on cultured dorsal root ganglion neurones (Shen and Crain, 1989, Werz and MacDonald, 1982), but lower, nanomolar, concentrations had the opposite effect (Shen and Crain, 1989).

Lombard and Besson (1989) tried to assess the relative importance of pre- and postsynaptic actions of morphine by recording the firing of dorsal horn neurones in decerebrate spinal rats with intact dorsal roots (and an induced peripheral arthritis) and comparable cells in non-arthritic animals with sectioned dorsal roots. Morphine (2mg kg^{-1} iv.) depressed the spontaneous firing of neurones in both preparations but a greater effect was observed in the arthritic animals with intact dorsal roots. Such a result indicates an action by opiates on the pathway from primary afferent terminals to the neurones studied but cannot distinguish between

effects on terminals and effects on interneurons interposed between such terminals and the neurones studied.

Histological studies.

Is there anatomical evidence for opiate mediated presynaptic inhibition at primary afferent terminals? Capsaicin pretreatment in newborn rats (Gamse et al, 1979a) and dorsal root transection in the primate (Lamotte et al, 1976) were both shown to produce a ~~small (10-20%)~~ decrease in the number of opiate binding sites in the SG. These results might indicate the existence of opiate receptors located presynaptically on a subpopulation of primary afferent terminals. More recent ultrastructural analyses (Cho and Basbaum, 1988,1989) failed to identify significant numbers of opioid peptide containing axo-axonic contacts to primary afferent structures in the spinal cord, thus failing to support the concept of a significant presynaptic function of opiates at such sites. (It is interesting to note that, in man, there is a marked reduction in morphine requirement for analgesia with increasing age (Moore et al, 1990), and thus the in vivo pattern of endogenous opiate function may be different in neonates versus immature versus adult animals. Such observations may underlie the inconsistencies outlined above).

Conclusion.

It has been the conclusion of studies which have observed reductions in tachykinin release with high concentrations of opiates that a presynaptic action on the central terminals of nociceptors is an important component of opiate analgesia (Yaksh and Noueihed, 1985). The results of the present experiments, considered with the

earlier findings of Kuraishi et al (1983) and Morton et al (1990) suggest that such an action does not occur significantly on the subpopulation of primary afferent fibres releasing tachykinin peptides. Primary afferent transmission mediated by the release of other compounds such as glutamate (where it does not coexist in primary afferent terminals with tachykinins, see Battaglia and Rustioni, 1988, de Biasi and Rustioni, 1988, p29) might, however, be impaired by the action of morphine in the spinal cord. It should be noted that the functional roles of the different opiate receptor types are clearly complex and have not yet been well established; this might have implications for the interpretation of results given here.

One recent study which highlights the problems arising in the interpretation of results obtained with ligands, such as morphine, which act at more than one receptor type (Kosterlitz et al, 1985) is that of Suarez-Roca et al (1992). This group reported a multiphasic effect of morphine on SP release from slices of rat trigeminal nucleus in vitro. Increasing doses of morphine in the bath solution, (10^{-9} , 10^{-7} , 10^{-6} , and 10^{-5} mM) respectively lowered, raised, lowered and raised K^{+} -evoked SP release. The simplest interpretation of this result must be that varying proportions of the different opioid receptor types; mu, delta and kappa, are activated with the different doses of morphine used. Potentiation, by morphine, of basal SP release from guinea-pig brain slices in vitro has also been reported (Chahl, 1990). Further studies on the modulation of tachykinin release from primary afferent terminals by receptor specific opioid peptides may ultimately prove to be rewarding: this is discussed more fully in the concluding section of this thesis.

SECTION 4.

THE EFFECTS OF NORADRENALINE AND MEDETOMIDINE ON NOXIOUS STIMULUS-EVOKED SUBSTANCE P RELEASE.

The results presented in this section have been published as:

"Microinjection of noradrenaline or medetomidine does not alter the pattern of nerve stimulus-evoked release of substance P in the spinal cord of the anaesthetised cat: a study with antibody microprobes." C.W.Lang, P.J.Hope, B.D.Grubb & A.W.Duggan.

British Journal of Pharmacology (1994), vol. 112, pp951-957.

The results presented here represent a major part of my studies, in which I was the principal investigator.

4.1 INTRODUCTION.

Whereas opiate drugs are widely used in human therapy, α_2 adrenoceptor agonists such as xylazine (Booth, 1988, Robertson and Muir, 1983, Waterman et al, 1988), detomidine (Clarke and Taylor, 1986, Lindberg, 1986) and medetomidine (Clarke and England, 1989, Jalanka, 1990, MacDonald et al, 1988, Vaha-Vahe, 1990, Vainio and Vaha-Vahe, 1990) have been more widely used in veterinary medicine, where their hypotensive effects are less marked (Nolan and Erhardt, 1990) and they are therefore safer to use than in man.

The history of α_2 adrenoceptor agonists in the treatment of pain is somewhat unusual in that interest in their clinical potential initially centred on the use of the drug clonidine in man. Clonidine has been administered to human patients in a variety of ways; orally, transdermally and epidurally, as part of both analgesic and preanaesthetic regimes (Bernard et al, 1991, Lilja et al, 1991, Ota et al, 1992, Rostaing et al, 1991, Segal et al, 1991, Tamsen and Gordh, 1984, Van Essen et al, 1991, Wright et al, 1990) but its efficacy in such treatments in man seems to be highly variable and given its hypotensive properties it has never become widely popular in human therapy. Curiously, the use of clonidine was never adopted by veterinary practitioners despite its reportedly greater potency and duration of action than, for example xylazine, when used as an epidural analgesic in domestic species such as the sheep (Nolan et al, 1987).

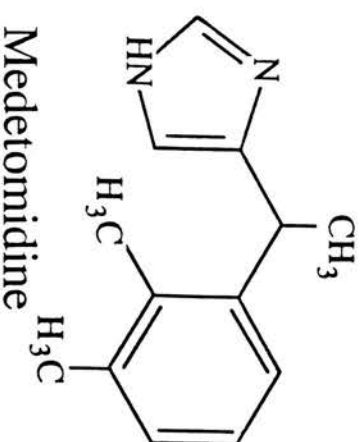
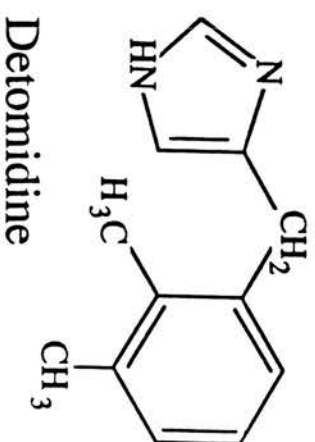
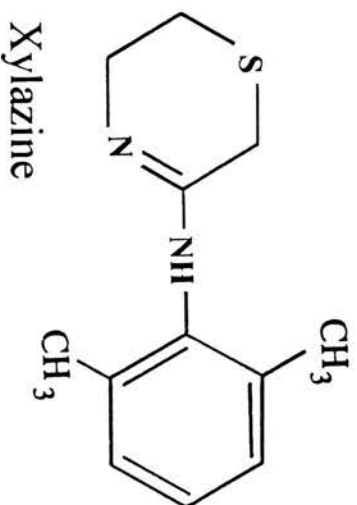
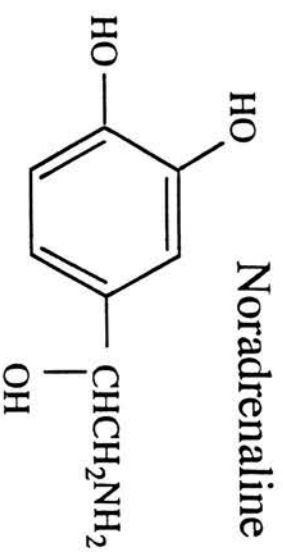
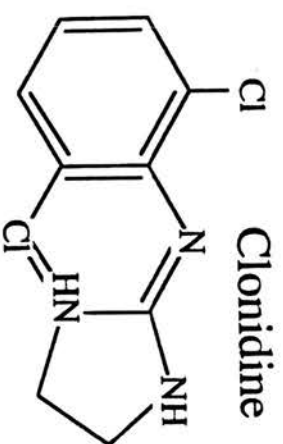
In recent years a number of imidazoline-derivative α_2 -selective agonist drugs have been developed. In order of their appearance these are xylazine, detomidine and medetomidine, all of which are

structurally related (see **figure 21**), and have found wide popularity in veterinary therapy principally as sedatives/analgesics (see above references and also Hall and Clarke, 1991, for review of therapeutic techniques), where one great advantage in their use is the reversibility of their effects either by classical α_2 adrenoceptor antagonists such as yohimbine and idazoxan (Docherty et al, 1987, Hsu et al, 1989) or by newer imidazoline derived compounds, notably atipamezole (Jalanka, 1990, Vaha-Vahe, 1990, Vainio and Vaha-Vahe, 1990). These drugs are less hypotensive than older α_2 agonists such as clonidine, and the most recent drug in the group to be developed, dexmedetomidine (simply the D-isomer of medetomidine) is now beginning to receive increased attention as a potential therapeutic agent in man (Aantaa et al, 1991, Aho et al, 1992, Karhuvaara et al, 1991, Sullivan et al, 1992, Scheinin et al, 1992). This has stimulated renewed interest in the role of α_2 adrenoceptor agonist drugs in animal models of spinal nociceptive processing.

Although all of the α_2 adrenoceptor agonist drugs used clinically (including the imidazoline derivatives) also show some agonist action at α_1 adrenoceptors, the results of studies with intrathecally applied receptor selective agonists and antagonists (Fleetwood-Walker et al, 1985, Headley et al, 1978, Howe et al, 1983) indicate that the antinociceptive properties of such drugs are selectively mediated via the α_2 receptor. The sedative properties of these drugs, produced in the brainstem, has also been attributed principally to their action at α_2 adrenoceptors (Schaffer et al, 1986, Vainio and Vaha-Vahe, 1990).

FIGURE 21. Molecular structure of five α_2 adrenoceptor agonist drugs, noradrenaline, clonidine and three structurally related imidazoline derivatives; xylazine, detomidine and medetomidine.

FIGURE 21.
MOLECULAR STRUCTURE OF SELECTED α_2 -ADRENOCEPTOR AGONISTS



A complication in studies on the actions of α_2 adrenoceptor agonist drugs in neural tissues is the discovery that many of them, especially the imidazoline derivatives, bind to a previously unrecognised group of receptors (Convents et al, 1989, Kamisaki et al, 1990, Michel et al, 1989). These receptors were originally recognised as non-adrenergic idazoxan binding sites, or NAIBS, and are now also termed 'imidazoline receptors' of which at least two, and possibly three subtypes can be identified (Michel and Insel, 1989, Wikberg et al, 1991). The contribution of spinal imidazoline binding sites to the effects of some of the newer drugs may prove to be quite dramatic: Savola and Savola (1992) reported that, as evaluated by displacement studies, at least 50% of dexmedetomidine binding in rat spinal cord could be attributed to NAIBS. It is difficult to evaluate the functional significance of these receptors as no truly specific agonists or antagonists for the imidazoline receptors have yet been developed, and it is therefore only possible to compare the effects of compounds with relatively higher or lower affinities for this receptor group. The results of one such study of this nature by Downie et al (1991) suggested that the effects of clonidine on sacral spinal reflexes may involve only α_2 adrenoceptors and not the imidazoline binding sites, but beyond this our understanding is still limited.

Although there is considerable data on the brainstem sites of origin of descending pathways controlling spinal transmission of nociceptive information, comparatively little is known about the mechanisms operating at the spinal end of these pathways. A wealth of evidence suggests that release of the monoamines, noradrenaline and 5-hydroxytryptamine (5HT), mediates in part the brainstem control of,

and segmental mechanisms active on, spinal nociceptive transmission. This evidence includes the effects of agonists (and antagonists) applied, in a variety of experimental models, either systemically (rat :Austin et al, 1976, Kayser et al, 1992, rabbit: Clarke et al, 1988, man: Nance et al, 1989), topically to the spinal cord (mouse: Hylden and Wilcox, 1983, rat: Camarata and Yaksh, 1985, Danzebrink and Gebhart, 1990, Fisher et al, 1991, Gordh et al, 1989, Hylden et al, 1991, Kuraishi et al, 1985b, Reddy et al, 1980, Sagen and Proudfit, 1984, Solomon and Gebhart, 1988a,b, Sullivan et al, 1987, 1992, Takano and Yaksh, 1991, Tjolsen et al, 1990, Tseng and Tang, 1989, Uhlen et al, 1990, cat: Collins et al, 1984, Nakagawa et al, 1990, sheep: Waterman et al, 1988) or near to single neurones in the dorsal spinal cord (cat: Calvillo and Ghignone, 1986, Curtis et al, 1983, Davies, 1989, Davies and Quinlan, 1985, Fleetwood-Walker et al, 1985, Headley et al, 1978, Jeftinija et al, 1981, Zhao and Duggan, 1988) and the behavioural effects, or electrophysiological changes in spinal cord neurones, produced by electrical stimulation of brain stem areas exhibiting monoamine containing nerve fibres (rat: Reynolds, 1969, cat: Gebhart et al, 1984, Mayer and Liebeskind, 1974, Oliveras et al, 1974).

In addition, a dense plexus of catecholamine containing nerve terminals (rat: Rajaofetra et al, 1992, Schroder and Skagerberg, 1985, cat: Doyle and Maxwell, 1991a,b, Lackner, 1980, primate: Chung et al, 1989, Westlund et al, 1984) and a marked density of catecholamine binding sites (rat: Giron et al, 1985, Jones et al, 1982, Unnerstall et al, 1984, Young and Kuhar, 1980, sheep: Bouchenaffa and Livingstone, 1987, man: Unnerstall et al, 1984) exists in the dorsal spinal cord, the main area of primary afferent

transmission, thus providing an anatomical basis for their involvement in the control of nociceptive transmission.

An attractive hypothesis for a mechanism of selective analgesia at the spinal level is a presynaptic reduction in transmitter release from primary afferent terminals of nociceptive origin. A body of work already exists investigating this possibility with regard to several analgesic compounds including noradrenaline. These include both in vivo and in vitro transmitter release studies (Kuraishi et al, 1985a, Ono et al, 1991, Pang and Vasko, 1986) and also electrophysiological assessment of the responses of individual nerve terminals in the dorsal spinal cord to drug application (Calvillo and Ghignone, 1986, Curtis et al, 1983, Jeftinija et al, 1981). Much of this earlier work produced controversial results, as will later be discussed, and the spinal mechanism of monoamine analgesia remained unresolved.

The most direct method of investigating a possible presynaptic reduction of neurotransmitter release by monoamines is to measure neurotransmitter release in the dorsal spinal cord, in response to a defined peripheral noxious stimulus, before and after monoamine application at the sites of neurotransmitter release. The tachykinin neuropeptide substance P (SP) is a neurotransmitter known to be associated with primary afferent fibres of nociceptive origin. The stimulus parameters for evoking the spinal release of SP have now been adequately defined (Duggan et al, 1988b) to make it a suitable transmitter for studies of this nature. The present series of experiments have used antibody microprobes to measure the release of immunoreactive (ir) SP in the superficial dorsal horn in response to peripheral noxious stimulation. The effects of noradrenaline microinjection from a micropipette with its tip positioned in the

region of the substantia gelatinosa (SG) have been evaluated in terms of any change in the pattern of stimulus evoked irSP detection. Because of the considerable evidence that the relevant receptor mediating the antinociceptive action of spinal noradrenaline is of the α_2 adrenoceptor type (Fleetwood-Walker et al, 1985, Headley et al, 1978, Howe et al, 1983), the effects of the highly selective α_2 adrenoceptor agonist medetomidine hydrochloride (supplied by Farnos, Finland) were also studied.

4.2 ANTIBODY MICROPROBE PREPARATION.

Antibody microprobes were prepared as described in the earlier section on general methodology. A polyclonal antiserum, raised against the carboxyl terminus of SP was used (Peninsula). Information supplied by the manufacturer indicated that this antiserum bound SP and fragments of SP containing at least the last five C-terminal amino acid residues (thus the major breakdown products of SP produced by the action of endopeptidases in vivo were unlikely to be detected), but showed negligible cross reactivity with either neurokinin A (NKA) or neurokinin B (NKB). This was verified by in vitro tests of the type outlined earlier.

4.3 ANIMAL PREPARATION/MICROPROBE ANALYSIS.

Experiments were performed on 7 cats, anaesthetised and subsequently prepared as described under the section on general methodology. All microprobes were inserted 4mm into the dorsal spinal

cord, which in this species places their tips in the upper ventral horn. All microprobes remained in the spinal cord for 10 minutes. The analysis of antibody microprobes subsequent to their use in vivo and in vitro also followed the methods outlined earlier.

4.4 STIMULUS PARAMETERS.

Peripheral noxious stimulation throughout a 10 minute period of antibody microprobe insertion was provided by electrical stimulation of the ipsilateral tibial nerve (groups of three 0.5ms pulses at 330Hz, repeated at 10Hz, amplitude 20V).

4.5 DRUG ADMINISTRATION AND EXPERIMENTAL REGIMES.

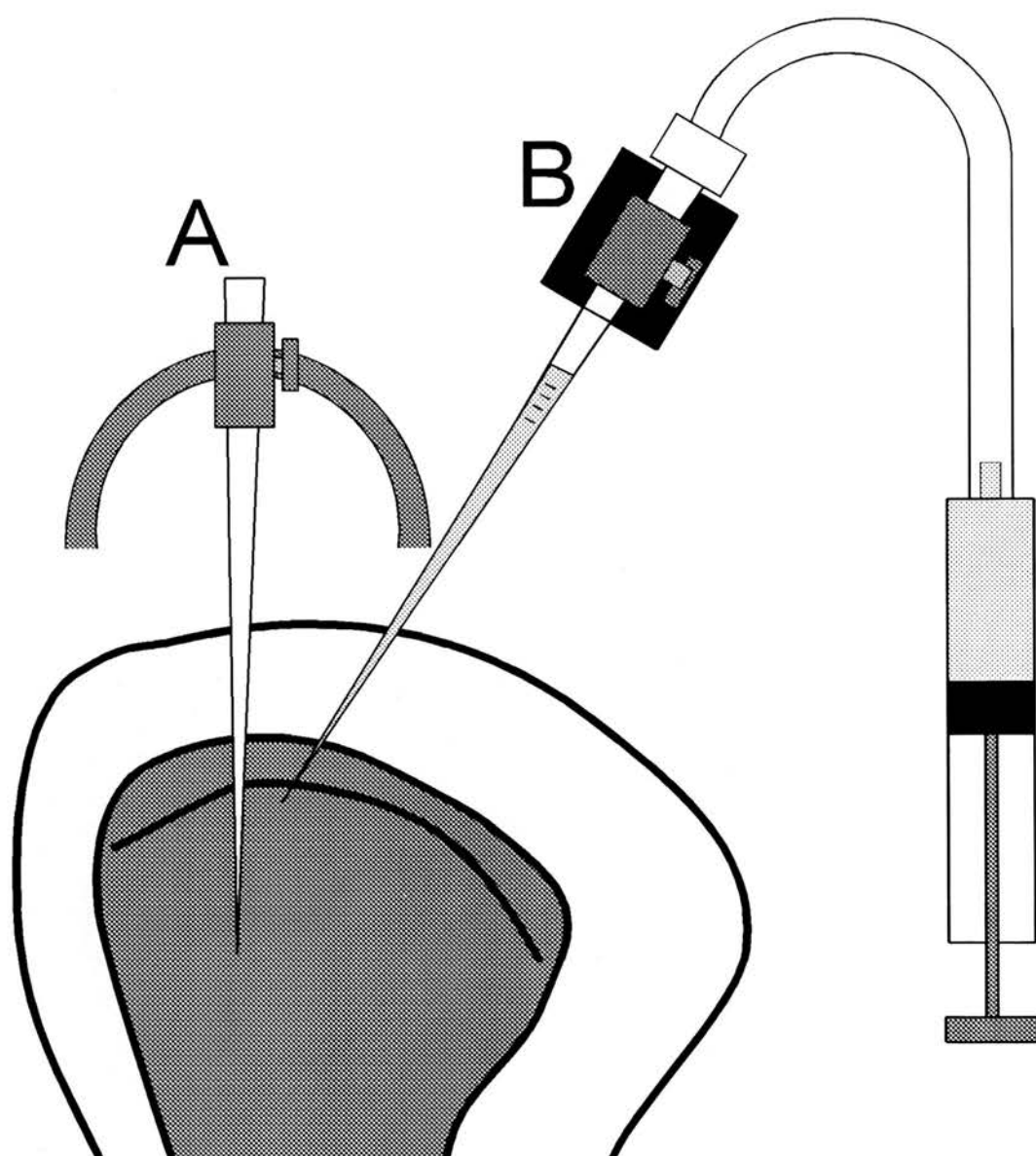
The technique of drug microinjection is illustrated in **figure 22**. Noradrenaline and medetomidine were microinjected (as $10^{-3}M$ solutions in sterile Ringer) into the dorsal horn from micropipettes (tip diameter 20-40 μm) positioned with a micromanipulator at subsequent depths of 1.0, 1.5, 2.0, and 2.5mm below the dorsal spinal cord surface. Although the peak release of irSP evoked by tibial nerve stimulation is in the superficial dorsal horn (1.1mm below cord surface, Duggan et al, 1988b) there is significant release in a zone extending approximately 0.5 to 1.0mm deep to this area. Hence the reason for performing microinjections at sites deep to the region of the substantia gelatinosa (SG).

FIGURE 22. Techniques of drug microinjection into the spinal cord. Drug solutions were pressure ejected from glass micropipettes of the same dimensions as those used to make antibody microprobes. Two general techniques were employed.

1) In the neuropeptide Y study (see section 5) some drug administration into the substantia gelatinosa was performed whilst single antibody microprobes were in situ. In this case two micromanipulators were used A. to position the antibody microprobe, held in a dorso-ventral plane and B. to position the drug filled micropipette, held at 10° from the dorso-ventral plane. Drug ejection was gradual, over a 10 minute period and was controlled by manually compressing the air held in the barrel of a 10ml glass syringe connected by tubing directly to the barrel of the drug filled pipette. Due to the very small tip diameter (20-40 μ m) of this micropipette, drug ejection could be performed in a very gradual manner and was monitored by following, under magnification, the meniscus of the drug solution moving between calibration marks placed beforehand on its barrel.

2) In all other cases drugs were microinjected prior to antibody microprobe insertion. This was performed as described above but either manipulator could be used. Each drug administration was typically of 0.2-0.5 μ l total volume, but divided into separate smaller injections, either at each of four depths (1.0, 1.5, 12.0, 2.5mm below cord surface) or at the same depth (1.1mm) but at multiple closely adjacent sites within the same pial patch.

FIGURE 22.
MICROINJECTION OF DRUG SOLUTIONS
INTO THE SPINAL CORD.



Individual micropipettes were calibrated by filling the shanks with firstly 0.2 μ l and then 0.4 μ l distilled water. These volumes were expelled from a Hamilton microlitre syringe onto a small piece of sterile Parafilm and rapidly sucked up into the micropipette under microscopic observation. Calibration marks were then drawn onto the surface of the micropipette with a fine marker pen, the water expelled under pressure and replaced in a similar manner with drug solution. The total drug volume was 0.5 μ l, ie. 0.125 μ l at each site. Previous experiments (Duggan et al, 1991a,1992, Schaible et al, 1992, (see also section 5) have shown that microinjection of Ringers solution or phosphate buffered saline (PBS) with comparable or larger volumes had no effect on the stimulus evoked release of irSP in this region of the spinal cord (see **figures 31** and **32** in later section). In the spinal cord, drug solutions were microinjected by means of pressure applied to the end of the micropipettes with a glass syringe and rubber tubing. This arrangement allowed very fine control over the ejection of solutions, which could be monitored under a binocular microscope.

The timing of microinjections in relation to stimuli evoking release of irSP is important in experiments of this type for several reasons. Firstly, if a compound is inactivated quickly the microinjection may need to be performed whilst a microprobe is already in situ and a stimulus being delivered. Previous experiments with neuropeptide Y (NPY) microinjection (Duggan et al, 1990, see section 5) and inhibitors of endopeptidase 24.11 (Duggan et al, 1992) showed that this was not necessary with these compounds. Secondly, the injected compound may need to diffuse within the spinal cord for an effect on irSP release to be detected; this implies a need to

inject a short time prior to testing for inhibition of release. In the experiments presented in this section injections of noradrenaline or medetomidine were performed after control microprobes (either no applied stimulus or nerve stimulus controls) had been removed from the cord, and microinjections were then administered gradually over a 5 minute period. Subsequent pairs of microprobes were then inserted as rapidly as possible into the cord and these were typically in situ 1-2 minutes following cessation of drug microinjection.

4.6 RESULTS.

4.6.1. STIMULUS-EVOKED RELEASE OF IMMUNOREACTIVE SUBSTANCE P.

The mean image analyses of 13 microprobes inserted 4mm into the spinal cord and left for 10 minutes in the absence of peripheral nerve stimulation ('Air controls') and 24 microprobes inserted for the same times and to the same depth but during electrical stimulation of myelinated and unmyelinated primary afferents of the ipsilateral tibial nerve ('Stim. controls') are illustrated in **figure 23A**. These microprobes are derived from the same individual experiments. Significant differences between these mean image analyses occur from 0.7 to 1.2 mm from the dorsal surface of the spinal cord, and there is also a smaller zone of significance approximately 1.5 mm below cord surface. The maximal difference occurs at a depth of 1.0 mm (**figure 23B**). This site of maximal

release approximates to the SG and lamina I and agrees with the findings of previous studies (Duggan et al, 1988b,1991a).

When dye is ejected from the tip of a single microprobe and its position in the spinal cord subsequently determined in a spinal cord section then sites of neuropeptide release can be inferred with great accuracy. With mean image analyses, as presented here, the location of sites of release is less precise. With microprobes placed a fixed distance into the spinal cord (4 mm in this series of experiments) the relationship of spinal laminae to distance from the tip will vary between individual animals and also, from microprobe to microprobe, with distance from the midline. When encoding the information describing the position of a microprobe, a correction factor is included when appropriate, but the referral of spinal laminae to a mean image analysis should still be regarded as approximate.

Summary. The noxious stimulus-evoked release of irSP was as expected from the findings of earlier studies employing the same techniques, with the site of maximal irSP release being centred in the SG of the spinal dorsal horn.

4.6.2. THE EFFECT OF NORADRENALINE MICROINJECTION ON NOXIOUS STIMULUS-EVOKED RELEASE OF IMMUNOREACTIVE SUBSTANCE P.

The mean image analyses of 38 microprobes inserted 4mm into the spinal cord for 10 minutes with concurrent electrical stimulation of myelinated and unmyelinated afferents of the ipsilateral tibial nerve ('stim. controls') and 35 microprobes inserted under the same conditions but immediately following the microinjection of 10^{-3}M noradrenaline at depths of 1.0, 1.5, 2.0 and 2.5mm below cord surface in the area of subsequent microprobe insertion ('post NA') are illustrated in **figure 24**. The two groups of microprobes are derived from the same individual experiments. The two analyses are virtually identical and at no point does the difference between their mean grey scale values attain statistical significance.

Summary. The microinjection of 10^{-3}M noradrenaline directly into the superficial dorsal horn of the spinal cord had no significant effect on the subsequent noxious stimulus-evoked release of irSP.

4.6.3.EFFECT OF MEDETOMIDINE MICROINJECTION ON NOXIOUS STIMULUS-EVOKED RELEASE OF IMMUNOREACTIVE SUBSTANCE P.

The mean image analyses of 31 microprobes inserted 4mm into the spinal cord for 10 minutes with concurrent electrical stimulation of the myelinated and unmyelinated afferents of the ipsilateral tibial nerve ('Stim. controls') and 20 microprobes inserted under the same conditions but following the microinjection of 10^{-3}M medetomidine at depths of 1.0, 1.5, 2.0 and 2.5mm below cord surface in the area of subsequent microprobe insertion ('post med.') are illustrated in **figure 25A**. These two groups are again derived from the same individual experiments, ie. the 'stim. controls' groups illustrated in **figures 24A** and **25A** do not represent the same microprobes.

Although these two mean image analyses do not appear to be as similar as those illustrated in **figure 24A**, there are again no statistically significant differences between their mean grey scale values at any point within the spinal cord (**figure 25B**). A small area of increased irSP detection following medetomidine microinjection is evident at the cord surface. This has been observed in previous studies following microinjection of sterile PBS-azide into the spinal cord (Duggan et al, 1991). It is thought to represent developing inflammation at the cord surface rather than a genuine pharmacological action of medetomidine.

Summary. The microinjection of 10^{-3}M medetomidine directly into the superficial dorsal horn of the spinal cord had no significant effect on the subsequent pattern of noxious stimulus-evoked irSP release within the spinal cord.

FIGURE 23. Nerve stimulus-evoked release of immunoreactive substance P in the superficial dorsal horn.

A. The mean image analysis of two groups of microprobes are plotted. Those which remained in the spinal cord for 10 minutes and without the application of a peripheral stimulus ('Air controls', n=13) and those present in the spinal cord during electrical stimulation of myelinated and unmyelinated afferents of the ipsilateral tibial nerve ('Stim. controls', n=24). The mean grey scale values of microprobes were calculated, as before, at 30 μ m intervals and a line joins these points. At each point the standard error of the mean (SEM), + for 'Stim. controls' and - for 'Air controls' is plotted.

B. A plot of the t-statistics derived from the differences of the means for the two groups of microprobes illustrated in A. This plot is superimposed on a diagram of a cross section of the lumbar spinal cord of the cat and the shaded area indicates where these differences attain significance ($t > 2$, $P < 0.05$).

FIGURE 23.
NORMAL PATTERN OF STIMULUS-EVOKED SUBSTANCE P RELEASE
(α_2 -ADRENOCEPTOR AGONIST STUDY)

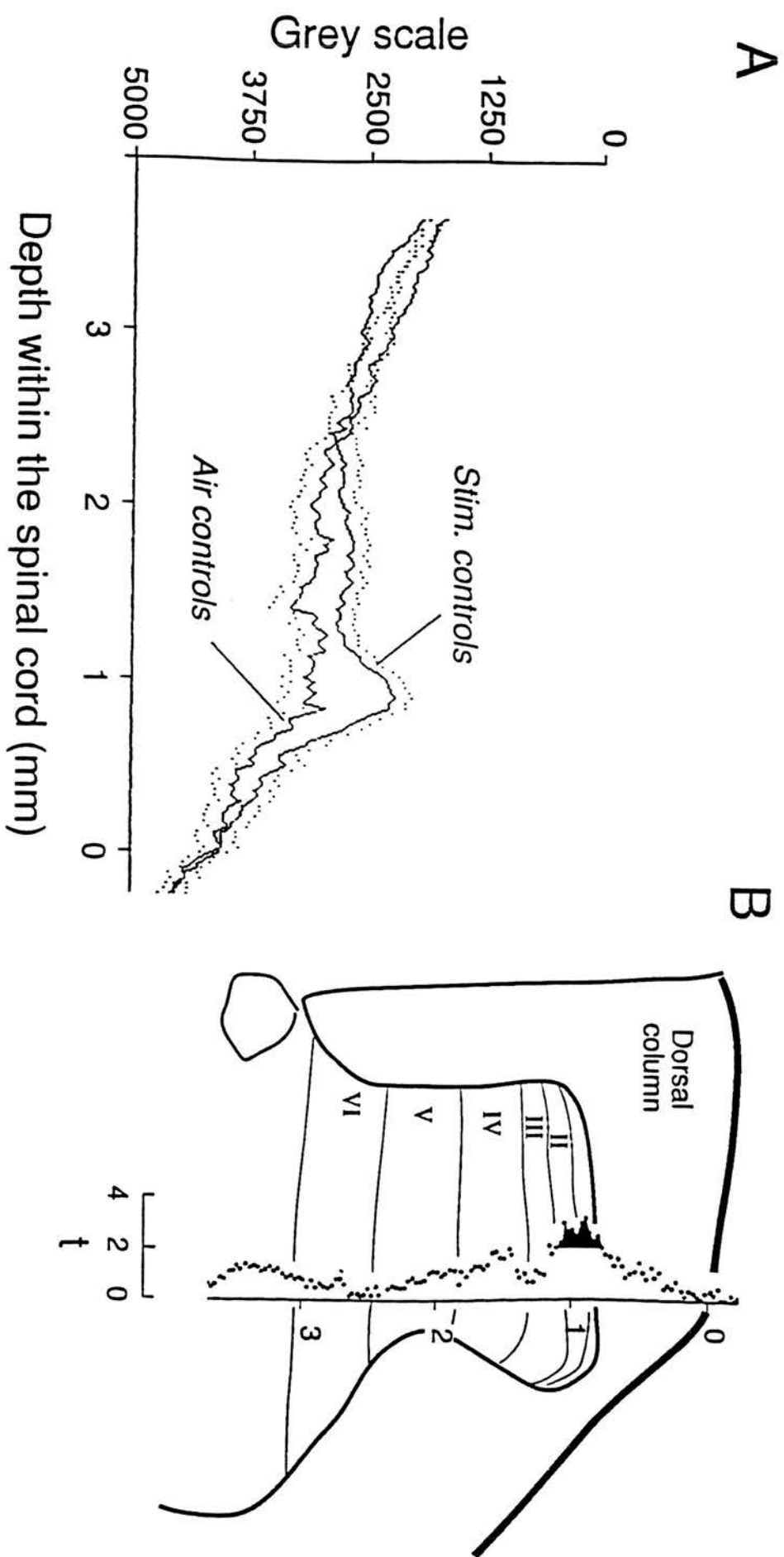


FIGURE 24. Lack of effect of microinjection of noradrenaline into the dorsal horn on the nerve stimulus-evoked release of immunoreactive substance P. The mean image analyses of two groups of microprobes are plotted: those present in the spinal cord for 10 minutes during electrical stimulation of the ipsilateral tibial nerve at C-fibre strength ('Stim. controls', n=38) and those with comparable stimulation, but inserted after microinjection of noradrenaline (10^{-3}M) into the dorsal horn ('Post NA', n=35). The format of the plot is similar to that in figure 23A.

FIGURE 24.
EFFECT OF NORADRENALINE ON STIMULUS-EVOKED LEVELS OF SUBSTANCE P.

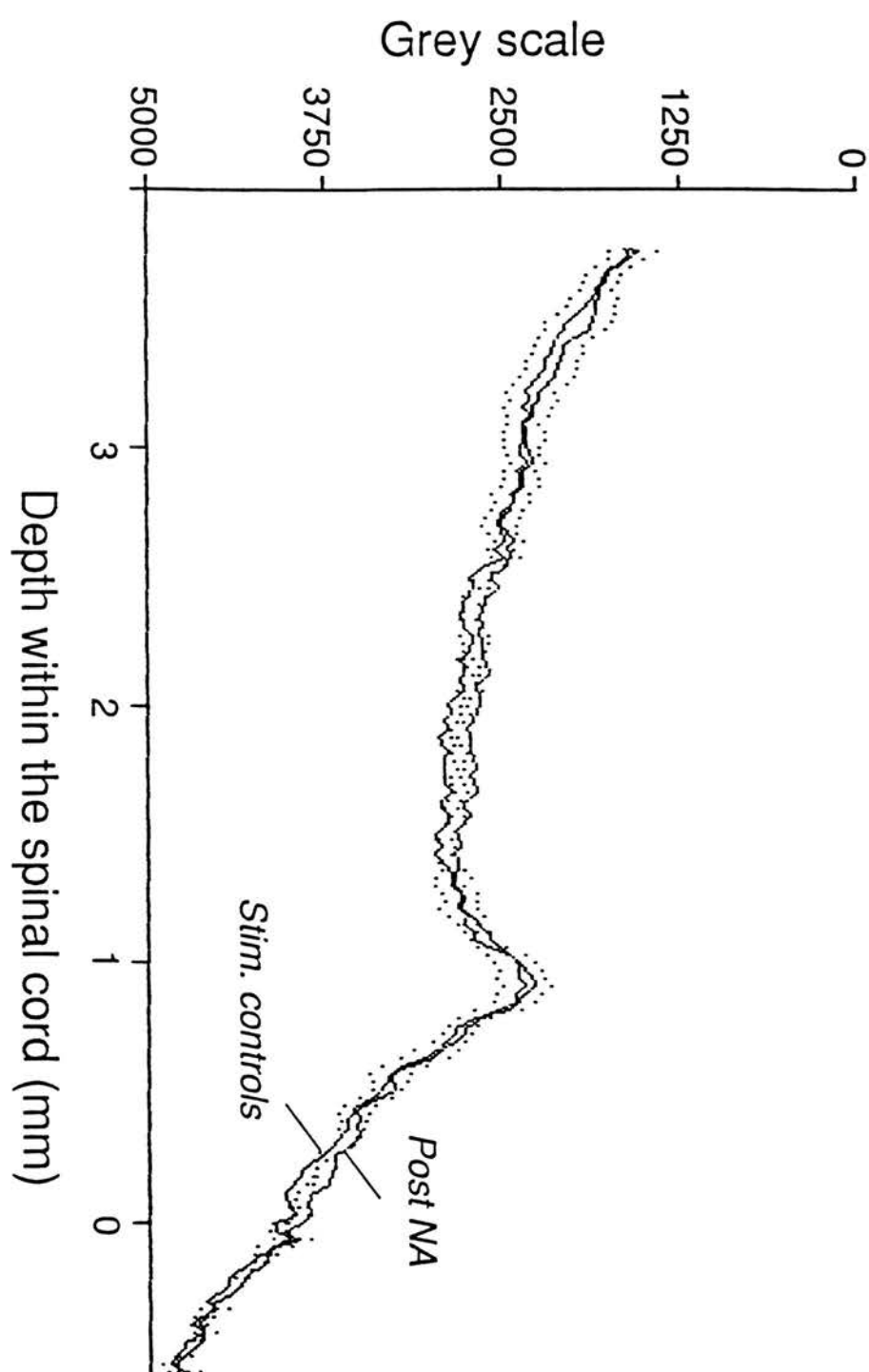
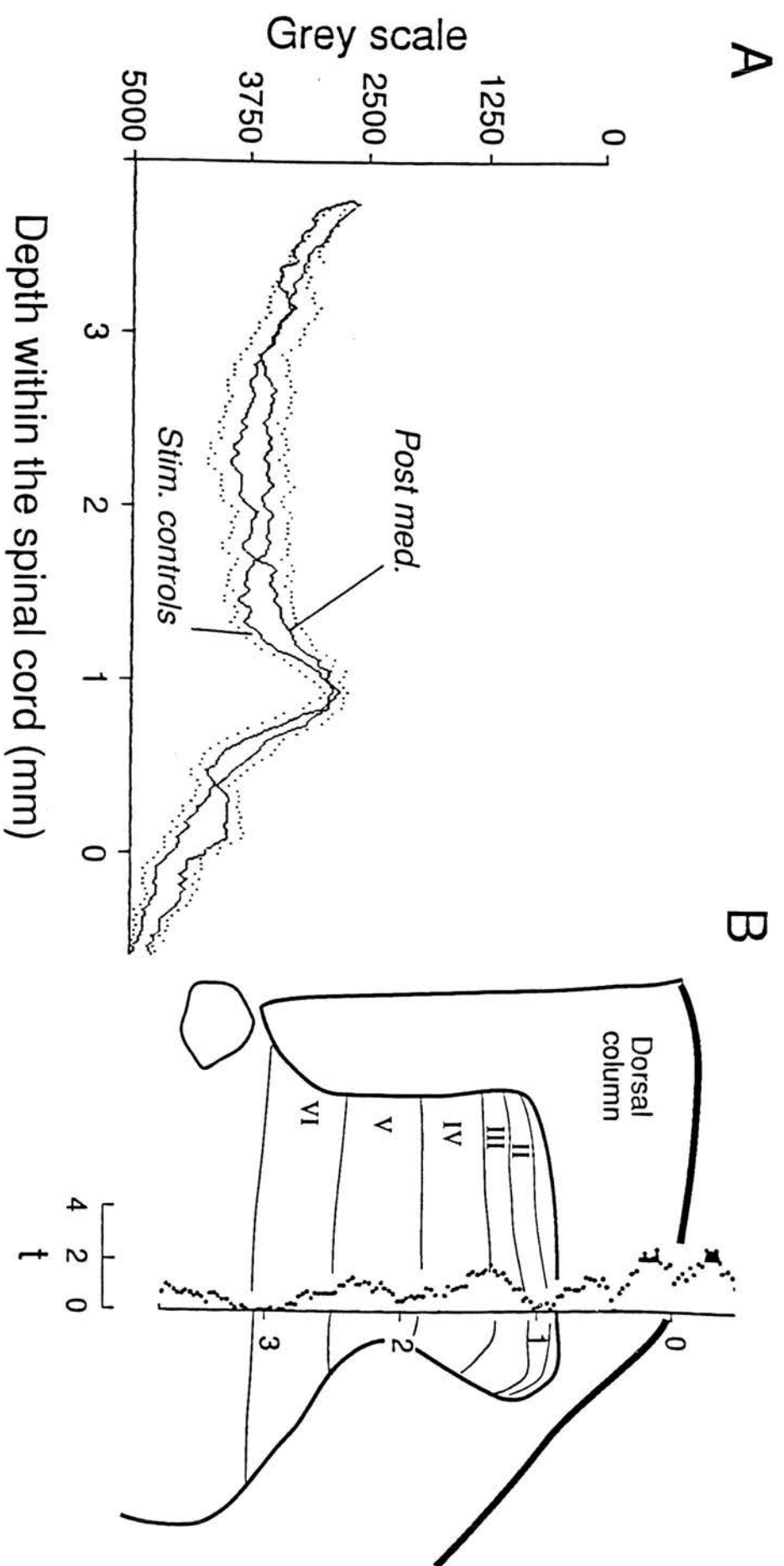


FIGURE 25. Lack of effect of microinjection of medetomidine into the dorsal horn on the nerve stimulus-evoked release of immunoreactive substance P.

A. The mean image analysis of microprobes present in the spinal cord for 10 minutes during electrical stimulation of the ipsilateral tibial nerve at C-fibre strength ('Stim. controls', n=31) and those with comparable stimulation, but inserted after microinjection of medetomidine (10^{-3}M) into the dorsal horn ('Post med.', n=20). The format of the plot is similar to that in figure 23A.

B. A plot of the t-statistics derived from the differences of the means of the two groups shown in A. is superimposed on a diagram of the lumbar spinal cord of the cat. The shaded areas indicate significant differences ($t > 2$, $P < 0.05$).

FIGURE 25.
EFFECT OF MEDETOMIDINE ON STIMULUS-EVOKED LEVELS OF SUBSTANCE P.



4.7 DISCUSSION.

The present experiments have shown that microinjections of noradrenaline (0.5 μ l, 10^{-3} M) or of the imidazoline derivative drug medetomidine (0.5 μ l, 10^{-3} M), which shows a high affinity for α_2 adrenoreceptors and imidazoline receptors, in the region of the SG of the cat spinal dorsal horn had no detectable effect on the nerve-stimulus evoked release of irSP.

Tachykinins and spinal nociceptive processing: interpretation of results.

Although it is improbable that the tachykinin peptides account for fast transmission from nociceptors (those impulses accurately signalling the onset, offset and intensity of a noxious stimulus), release of these compounds from the central terminals of nociceptors is well established (Duggan et al, 1988b,1989,1990a,b,1991,1992, Hope et al, 1990b,c, Lang et al, 1991,1994, Lang and Hope, 1994, Morton et al, 1990,1991, Schaible et al,1990,1992), and potential controls of such release require investigation.

Interpretation of the present results is largely based on the belief that, in acutely spinalised cats, the irSP detected in the spinal cord following peripheral nerve stimulation is largely of primary afferent origin. A contribution from intrinsic spinal neurones cannot be excluded, but release from fibres of supraspinal origin cannot have occurred in a spinalised preparation. It is worth noting that the distribution of irSP detection by antibody microprobes, following peripheral nerve stimulation, in these experiments is in accord with the findings of previous antibody

microprobe studies (Duggan et al, 1988b,1991, Schaible et al, 1992).

Microinjection of noradrenaline directly into the SG had no effect on the stimulus-evoked release of irSP. Administration of drugs in this manner has previously been shown to be an effective way of modulating stimulus evoked irSP release. Thus microinjection of NPY was shown to reduce irSP detection following peripheral nerve stimulation (Duggan et al, 1991, and see also section 5) whereas microinjection of calcitonin gene-related peptide (CGRP) or of the peptidase inhibitor drug, kelatorphan, enhanced post-stimulus detection of irSP (Duggan et al, 1992, Schaible et al, 1992). Noradrenaline may, following microinjection have been subject to rapid reuptake and degradation in the spinal cord. Medetomidine however, the other α_2 adrenoceptor agonist drug tested in these studies, does not appear to be subject to any reuptake mechanism (Dr.R.Virtanen, Farnos, personal communication), but instead is metabolised by hydroxylation in the liver (Salonen and Eloranta, 1990). Medetomidine should, therefore, remain active for some time following its injection into neuronal tissue, yet still failed to show any effect on irSP release. Similar arguments can be used in considering the ability of the drugs used to diffuse to appropriate sites of action in the spinal cord: medetomidine is highly lipophilic (Savola et al, 1986) and should readily spread throughout neuronal tissue following microinjection.

Other studies on α_2 adrenoceptor agonists and SP release.

The current observation that noradrenaline was ineffective in reducing noxious stimulus evoked irSP release is in conflict with the findings of some earlier studies. Ono et al (1991) demonstrated that

clonidine (10^{-5}M) and tizanidine (10^{-5}M), both α_2 adrenoceptor agonist drugs, reduced the veratridine-induced release of SP from slices of rat spinal cord in vitro. This effect was blocked by yohimbine (10^{-5}M) and also by prazosin (10^{-5}M), leading Ono and co-workers to conclude that the reduction of SP release was mediated by α_{2B} adrenoceptors. Pang and Vasko (1986) reported that noradrenaline (10^{-5}M) inhibited the high K^+ -evoked release of SP from a similar preparation of rat spinal cord, and this inhibition was reversed by phentolamine (10^{-5}M) indicating that the effect was mediated by receptors in the α adrenoceptor group.

Veratridine application, or simple elevation of extracellular K^+ levels, do not represent stimuli which are selective to structures of primary afferent origin, and perfusion of cord slices allows no spatial resolution of the source of neurotransmitter release. SP is also present in intrinsic neurones of the spinal cord and the SP detected in these in vitro studies may be derived in part from such structures: control mechanisms at spinal interneurones might be very different from those at primary afferent terminals. Lastly, Kuraishi et al (1985a) reported that noradrenaline (10^{-5}M) added to the perfusate of a push-pull cannula inserted into the upper dorsal horn of rabbit spinal cord in vivo reduced the release of irSP in response to a peripheral noxious mechanical stimulus. This effect was reversed by both yohimbine (10^{-4}M) and prazosin (10^{-4}M). The low group numbers in these experiments ($n=5$) and the very large variance in the release of irSP following the addition of antagonist drugs to the perfusate did, however, indicate the need to re-examine the effects of noradrenaline on stimulus evoked irSP release in vivo.

Cellular mechanisms of action of α_2 adrenoceptor agonists in blocking spinal nociceptive transmission; electrophysiological and histological studies.

These results are part of an extended study on possible presynaptic controls of tachykinin release from the central terminals of nociceptors. Presynaptic inhibition of transmitter release from such terminals has been proposed as being important in several mechanisms of analgesia, including that resulting from impulses arriving in large diameter afferents (Melzak and Wall, 1965) and analgesia resulting from electrical stimulation in the mid-brain periaqueductal grey matter (Fields and Basbaum, 1978). There is, however, relatively little data bearing directly on this question.

Both noradrenaline (Fleetwood-Walker et al, 1985, Headley et al, 1978, Reddy and Yaksh, 1980) and medetomidine, or its D-isomer form dexmedetomidine, (Fisher et al, 1991, Ossipov et al, 1990b, Pertovaara et al, 1991) have been shown to block transmission of nociceptive inputs in the rat and cat at the level of the spinal cord, either by their actions when applied intrathecally, or by merit of an observable antagonism of their effects by intrathecally applied compounds. The actual mechanisms responsible for such inhibition of spinal nociceptive processing have, however, remained largely unresolved.

As in the previous section, the electrophysiological and anatomical evidence to support a presynaptic action of these compounds should be considered. Calvillo and Ghignone (1986) reported that intravenous clonidine, an α_2 adrenoceptor agonist, selectively increased the electrical excitability of C-fibre primary afferent terminals in cat spinal cord, and that this excitability was reversed

by both yohimbine and phentolamine. Such primary afferent depolarisation might lead to presynaptic inhibition; gamma-aminobutyric acid (GABA) produces a similar, depolarising, action at the central terminals of larger diameter (A-delta) primary afferent terminals, the mechanism thought to underly its inhibitory effects at such sites (Curtis and Lodge, 1982, Sastry, 1979a). However, Jeftinija et al (1981) have previously reported that micro-ionophoresed noradrenaline reduced electrical excitability in approximately 2/3 of C-fibre central terminals, perhaps indicating a novel, presynaptic inhibitory mechanism. In this earlier study the effects of noradrenaline could not be reversed with either phentolamine or yohimbine applied locally or systemically, and the exact significance of these findings remains unclear.

It is of note that in recent immunocytochemical studies, Doyle and Maxwell (1991a,b,1993b) failed to identify catecholamine-containing axo-axonic contacts in cat SG, yet axo-dendritic and axo-somatic contacts showing positive immunoreactivities for two of the enzymes involved in noradrenaline synthesis, tyrosine hydroxylase and dopamine- β -hydroxylase were readily identifiable. It is the former axo-axonic type of contact which is thought to be involved in presynaptic control at primary afferent terminals and these results do not, therefore, provide anatomical evidence to support a presynaptic action of noradrenaline at such sites. (The same group have, however, identified axo-axonic contacts in the cat SG containing NPY (Doyle and Maxwell, 1993a) which is of great significance in relation to the results presented in the next section). Wikberg and Hajos (1987) reported that primary afferent terminal destruction by capsaicin treatment in neonatal mice did not

alter the number of [3H]-clonidine binding sites in the spinal cord, again suggesting that spinal α_2 binding sites were located postsynaptically to primary afferent terminals. The pattern of catecholaminergic innervation in the spinal cord of another species of rodent, the rat, has been shown to change over a considerable period of time following birth (Commissiong, 1983) and the findings of Wikberg and Hajos might, therefore, be viewed with a degree of caution.

The roles of imidazoline receptors and of both α_1 and α_2 adrenoceptors in spinal cord processing.

Noradrenaline may have acted on more than one α adrenoceptor subtype in the dorsal horn. It is not known if α_1 adrenoceptors exist on the central terminals of nociceptors, but α_1 adrenoceptor-mediated effects have been observed on neurones of the SG, the major site of primary afferent fibre termination. Thus North and Yoshimura (1984) observed a prazosin (α_1) reversible depolarisation of SG neurones in a slice preparation of rat dorsal horn but also a yohimbine (α_2) reversible hyperpolarisation of these cells. If these differing actions occurred simultaneously on nociceptive terminals following noradrenaline microinjection, then no net effect on irSP release might be detectable. Medetomidine is, however, a highly α_2 adrenoceptor selective drug, approximately 1600 times higher affinity for the α_2 as compared to the α_1 adrenoceptor subtype (Virtanen et al, 1988), yet it also failed to alter irSP release. This suggests that there is no α_2 adrenoceptor mediated inhibition of stimulus evoked irSP release from primary afferent terminals of nociceptive origin.

As discussed earlier, the physiology of imidazoline receptor-mediated effects in the spinal cord is currently poorly understood, largely due to the current lack of any imidazoline receptor-specific agonist or antagonist drugs. As detailed earlier, it has recently been suggested that at least 50% of the binding of labelled forms of drugs like medetomidine in the spinal cord is accounted for by binding at non-adrenergic sites (Savola and Savola, 1992, see also concluding section of thesis). It is likely, therefore, that in the present series of experiments medetomidine was active at a population of imidazoline receptors in the cat spinal cord, in addition to its effects on α_2 adrenoceptors. The present finding, that medetomidine was without effect in reducing noxious stimulus-evoked release of irSP does not support a presynaptic inhibition of transmitter release from primary afferent terminals mediated by either α_2 adrenoceptors or imidazoline receptors.

One criticism which might be levelled at the protocol used in the current series of experiments is the lack of any antagonist drug administration. This was quite deliberate. If an inhibition of stimulus-evoked irSP release had been detected in response to microinjection of either drug, further experiments would have been performed in an attempt to block such inhibition with either the α_2 antagonist yohimbine, or the imidazoline derivative atipamezole, which is active as an antagonist at both α_2 and imidazoline receptors. Such extended studies would have allowed classification of any effect as being either α_2 , or non- α_2 adrenoceptor mediated. In the case of medetomidine, but not of noradrenaline, producing inhibition of irSP release, use of the antagonists available would have gone some way to determining whether this effect was due to the

higher potency and selectivity of medetomidine for the α_2 class of adrenoceptor, or alternatively a consequence of its affinity for imidazoline receptors. Given the results obtained, such extended studies would have been of little value.

Conclusion.

In conclusion, the results presented in this section indicate that the spinal analgesia produced by noradrenaline and medetomidine is in neither case mediated via presynaptic inhibition of irSP release from the central terminals of C- or A-delta primary afferent fibres. In accord with the results of electrophysiological and histological studies by other groups, my experiments suggest that the well-documented effects of noradrenaline in the superficial dorsal horn result from postsynaptic effects on intrinsic neurones of this area. In the case of medetomidine this conclusion can be applied both to its effects mediated via α_2 adrenoceptors and its presumed, but as yet unidentified, actions mediated via imidazoline receptors.

<p style="text-align: center;"><u>SECTION 5.</u></p> <p style="text-align: center;"><u>THE EFFECT OF NEUROPEPTIDE Y ON</u></p> <p style="text-align: center;"><u>NOXIOUS STIMULUS-EVOKED SUBSTANCE P RELEASE.</u></p>

These results have previously been published as:

"Microinjection of neuropeptide Y into the superficial dorsal horn reduces the stimulus-evoked release of immunoreactive substance P in the anaesthetised cat." A.W. Duggan, P.J. Hope & C.W. Lang.

Neuroscience (1991) volume 44, pp 733 - 740.

The results presented in this section are derived from experiments in which I collaborated equally with the researchers named above.

5.1 INTRODUCTION.

Neuropeptide Y (NPY) is a 36 amino acid peptide found throughout the central nervous system of many species. The superficial dorsal horn of the spinal cord of several mammalian and non-mammalian species has been shown to contain a dense network of NPY-containing fibres, mainly of supraspinal origin (man: Allen et al, 1984, rabbit: Blessing et al, 1987, elasmobranch: Cameron et al, 1990, rat: Chronwell et al, 1985, Henschen et al, 1988, cat: Krukoff, 1987). NPY is also present in the terminals of some primary afferent fibres (Wakisaka et al, 1991, 1992), although not significantly in C-fibre afferents. The superficial dorsal horn of the spinal cord is a site of potential interaction of many neuroactive compounds. The substantia gelatinosa (SG) is the major site of termination of cutaneous unmyelinated primary afferents (Light and Perl, 1977), many of which are nociceptors, and these fibres contain excitatory amino acids and neuropeptides, either singly or in a complex series of combinations (Duggan and Weihe, 1990). In addition, both intrinsic neurones of the SG and the terminals of propriospinal and supraspinally derived fibres contain amino acids, monoamines and neuropeptides. To understand the interactions between such a large number of compounds is clearly a complex task. One aspect which can be investigated experimentally is whether any of the substances contained within the SG act to reduce the release of compounds from the central terminals of primary afferent fibres, in the manner which has already been outlined in relation to α_2 adrenoceptor agonists and opioids in the preceding sections. In this series of experiments, however, the choice of compound, NPY, represents an agent which

although known to be present throughout the peripheral (PNS) and central nervous systems (CNS) (indeed it is one of the most ubiquitous CNS peptides) has not yet been well defined in terms of its physiological role. Consequently the potential of NPY, or of agents acting at NPY receptors, as new analgesic agents has not yet been fully investigated.

In both the PNS and the CNS there are several instances where NPY has been shown to have effects on nerve terminals, *in vivo* and *in vitro*, consistent with an impairment of transmitter release from those terminals (hippocampal slice: Colmers et al, 1991, Hass et al, 1987, cardiac vagal fibres: Kilborn et al, 1985, peripheral nerve terminals, Guiliani et al, 1989, Wahlestedt et al, 1986).

It has already been mentioned that an inhibition of transmitter release from the central terminals of nociceptors has been used to explain several mechanisms of analgesia. Examples include the 'Gate Theory' of Melzak and Wall (1965), and the hypothesis of Jessell and Iversen (1977) which evolved from their study of opiate effects on substance P (SP) release from slices of rat trigeminal nucleus. (This topic has already been discussed at some length, see pages 70 - 75, and has been reviewed in part by both Basbaum and Fields, 1984, and Duggan, 1985). If NPY had such an action in the superficial dorsal horn, and if the relevant receptors were located near the central terminals of primary afferent fibres of nociceptive origin, then such a mechanism could contribute to analgesia of both segmental and supraspinal origin. Both mechanisms merit investigation following reports of analgesic effects following the intrathecal administration of NPY in the rat (Hua et al, 1991, Yaksh, 1990).

The series of experiments presented in this section were performed in order to test the effects of NPY, microinjected directly into the dorsal spinal cords of anaesthetised cats on the subsequent release pattern of immunoreactive (ir)SP seen following electrical stimulation of afferent fibres of presumed nociceptive origin. The experimental protocol (outlined on the following pages) was similar to that used in studies of noradrenaline/medetomidine modulation of stimulus-evoked irSP release (presented in section 4), thus allowing a direct comparison to be made between the two series of experiments.

5.2 ANTIBODY MICROPROBE PREPARATION.

Antibody microprobes were prepared as described in the earlier section on general methodology, with the exception that the protein A used to bind immunoglobulins onto the microprobe surfaces was produced by Porton, not in this instance by Sigma. A polyclonal antiserum, raised against the carboxyl terminus of SP was used (Peninsula). This was the same antiserum as that subsequently used in the series of experiments presented in section 4 (see page 143). Information supplied by the manufacturer indicated that this antiserum bound SP and fragments of SP containing at least the last five C-terminal amino acid residues, (thus the major breakdown products of SP produced by the action of endopeptidases in vivo were unlikely to be detected), but showed negligible cross reactivity with either neurokinin A (NKA) or neurokinin (NKB). This was verified by in vitro tests as previously described.

5.3 ANIMAL PREPARATION/MICROPROBE ANALYSIS.

Experiments were performed on seven cats, anaesthetised and subsequently prepared as described under the section on general methodology. All microprobes were inserted 3mm into the dorsal spinal cord, which in this species places their tips in the upper ventral horn. All microprobes remained in the spinal cord for 20 minutes. The analysis of antibody microprobes subsequent to their use in vivo and in vitro also followed the methods outlined earlier.

5.4 STIMULUS PARAMETERS.

Peripheral noxious stimulation throughout a 20 minute period of antibody microprobe insertion was provided by electrical stimulation of the ipsilateral tibial nerve (groups of three 0.5ms pulses at 330Hz, repeated at 10-30Hz (constant in each experiment), amplitude 20V).

5.5 DRUG ADMINISTRATION AND EXPERIMENTAL REGIMES.

Microinjection (see **figure 22**) of NPY was performed in two ways. In two experiments the tips of an antibody microprobe and a micropipette containing 0.5 μ l of NPY were positioned just above the dorsal surface of the spinal cord. The microprobe was held vertically in a micromanipulator and the micropipette was held in a separate micromanipulator angled at 10° from vertical in an anteroposterior

plane. The microprobe was inserted 3mm into the spinal cord. Using basic trigonometry, the distance the micropipette needed to be moved to position the tip 1.1mm below the dorsal surface of the spinal cord was calculated (1.12mm). The micropipette was then inserted to this distance, thus placing the tip approximately in the middle of the SG. NPY was subsequently injected slowly throughout the entire 20 minute period of microprobe insertion to a total volume of approximately 0.2 μ l.

In the remaining five experiments, a series of two to three microinjections, each of approximately 0.2 μ l, was made around the margins of an area of denuded pia mater (ie. at the edges of the pial patches) and following this microinjection of NPY pairs of antibody microprobes were inserted into the centre of the patches. NPY was microinjected at a concentration of 10^{-7} M in sterile phosphate buffered saline (PBS) in one experiment and 10^{-5} M in sterile PBS in six experiments.

The method of calibrating and filling micropipettes was discussed in section 4, as was consideration of the timing of drug microinjection relevant to antibody microprobe insertion. The need for vehicle/volume controls was also discussed in section 4, but it was as part of this study on NPY microinjection that extensive controls involving the microinjection of PBS alone into the dorsal spinal cord were performed. The results of these controls are presented here.

5.6 RESULTS.

5.6.1 STIMULUS EVOKED RELEASE OF IMMUNOREACTIVE SUBSTANCE P.

The mean image analysis of 16 microprobes inserted 3mm into the spinal cord and left in situ for 20 minutes in the absence of any form of applied peripheral stimulus ('Air controls') and 33 microprobes inserted for the same time and to the same depth but during electrical stimulation of both the unmyelinated and the myelinated afferent fibres of the ipsilateral tibial nerve ('Nerve stim.') are illustrated in **figure 26A**. These microprobes were derived from all seven experiments in this study.

Significant differences between these mean image analyses occur from 0.6 to 1.4mm below the dorsal surface of the spinal cord with the maximal difference at a depth of 1.0mm as shown in **figure 26B**. This site of maximal irSP release approximates to the location of the substantia gelatinosa (SG) and lamina I of the dorsal grey matter and thus agrees with the findings of other studies (Duggan et al, 1988b, 1991a, Lang et al, 1994). As with these other studies, and for the same reasons, the referral of spinal laminae to such mean image analyses should be considered approximate.

Summary. The noxious stimulus-evoked release of irSP was as expected from the findings of earlier studies employing the same techniques, with the site of maximal irSP release being centred in the SG of the spinal dorsal horn.

5.6.2 THE EFFECT OF CONCURRENT NPY MICROINJECTION
ON NOXIOUS STIMULUS-EVOKED RELEASE OF
IMMUNOREACTIVE SUBSTANCE P.

The mean image analyses of 12 microprobes inserted 3mm into the spinal cord for 20 minutes and with concurrent electrical stimulation of the unmyelinated and myelinated afferents of the ipsilateral tibial nerve ('Stim. controls') and 13 microprobes inserted under the same conditions but with NPY (10^{-7}M in one experiment, 10^{-5}M in one other) being microinjected 1.1mm below the dorsal surface of the spinal cord and approximately $100\mu\text{m}$ from the microprobe shaft ('During NPY ') are compared in **figure 27**. Although the mean image analysis of microprobes inserted during NPY microinjection is displaced below that of control microprobes (indicating reduced stimulus-evoked release of irSP), this difference only marginally attains statistical significance over a very restricted area approximately 1.5mm below cord surface.

Summary. Injection of NPY concurrent with tibial nerve stimulation/microprobe insertion resulted in only a marginal decrease in the levels of irSP detected.

5.6.3 THE EFFECT OF PRIOR NPY MICROINJECTION ON NOXIOUS STIMULUS-EVOKED RELEASE OF IMMUNOREACTIVE SUBSTANCE P.

The effectiveness of ipsilateral tibial nerve stimulation in evoking release of irSP prior to and in the 20 minute period following NPY microinjection (10^{-5}M , five experiments) is compared in **figure 28**. The mean image analysis for 20 microprobes inserted following NPY microinjection ('0-20 min post NPY') is significantly displaced below that of 33 stimulus control microprobes ('Stim. controls') over an area beginning at 0.8mm below cord surface and extending to a depth of 2.0mm. In the second 20 minute period of tibial nerve stimulation after NPY microinjection the stimulus-evoked release of irSP is still reduced (20 microprobes, '21-40 min post NPY') but over a much more restricted area of the spinal cord. This is illustrated in **figure 29**, which shows a significant reduction in irSP detection ($t > 2$) only in the region of laminae I and II of the dorsal grey matter.

A small number of microprobes ($n=5$) were inserted during a third period of nerve stimulation. The mean image analysis of these 5 microprobes, not illustrated, was observed not to differ significantly from that of the pre-NPY microinjection control group.

Summary. Microinjection of 0.2-0.6 μl of NPY (10^{-5}M in PBS) inhibited the subsequent nerve stimulus-evoked release of irSP. This effect was time dependant, being maximal in the first 20 minutes following NPY microinjection, and ceasing (within the sensitivity of the technique used) by 41-60 minutes after drug administration.

5.6.4 RELEASE OF IMMUNOREACTIVE SUBSTANCE P
FOLLOWING MICROINJECTION OF PHOSPHATE-BUFFERED SALINE
CONTAINING SODIUM AZIDE.

Figure 30 illustrates the mean image analyses of 25 microprobes inserted into the spinal cord during ipsilateral tibial nerve stimulation but prior to microinjection of PBS-azide in similar volumes to those used for NPY microinjection ('Stim. controls') and of 12 microprobes inserted with the same peripheral stimulus but in the 0-20 minute period following microinjection ('0-20 min post PBS'). There is a small zone of enhanced irSP detection at the surface of the spinal cord following PBS microinjection, but within the spinal cord the two mean image analyses are virtually identical. (This zone of enhanced release of irSP on the spinal cord surface was also seen following medetomidine microinjection, as detailed in section 4 and, as in that study, is thought to represent a time-dependant inflammatory effect rather than a drug-mediated change).

Figure 31 compares the mean image analysis of the preinjection group of microprobes ('Stim. controls') with that of 7 microprobes inserted during ipsilateral tibial nerve stimulation, but 21-40 minutes after PBS-azide microinjection ('21-40 min post PBS'). Again the two mean image analyses are virtually identical.

Summary Microinjection of PBS containing azide, in similar volumes as those used for the microinjection of drug-containing solutions had no detectable effect on the subsequent nerve stimulus-evoked pattern of irSP release within the spinal cord.

FIGURE 26. Stimulus-evoked release of immunoreactive substance P.

A. 'Air controls', the mean image analysis of microprobes (n=16) inserted 3mm into the spinal cord for 20 minutes without any peripheral stimulation, compared with a second group, 'Nerve stim.' (n=33) present in the spinal cord for 20 minutes but during electrical stimulation of the ipsilateral tibial nerve at C-fibre strength. The mean grey scale values of microprobes were calculated, as before, at 30 μ m intervals and a line joins these points. At each point the standard error of the mean (SEM), + for 'Nerve stim.' and - for 'Air controls' is plotted.

B. A plot of the t-statistics derived from the differences of the means for the two groups of microprobes illustrated in A. This plot is superimposed on a diagram of a cross section of the lumbar spinal cord of the cat and the shaded area indicates where these differences attain significance ($t > 2$, $P < 0.05$).

FIGURE 26.
NORMAL PATTERN OF STIMULUS-EVOKED SUBSTANCE P
RELEASE (NPY STUDY) .

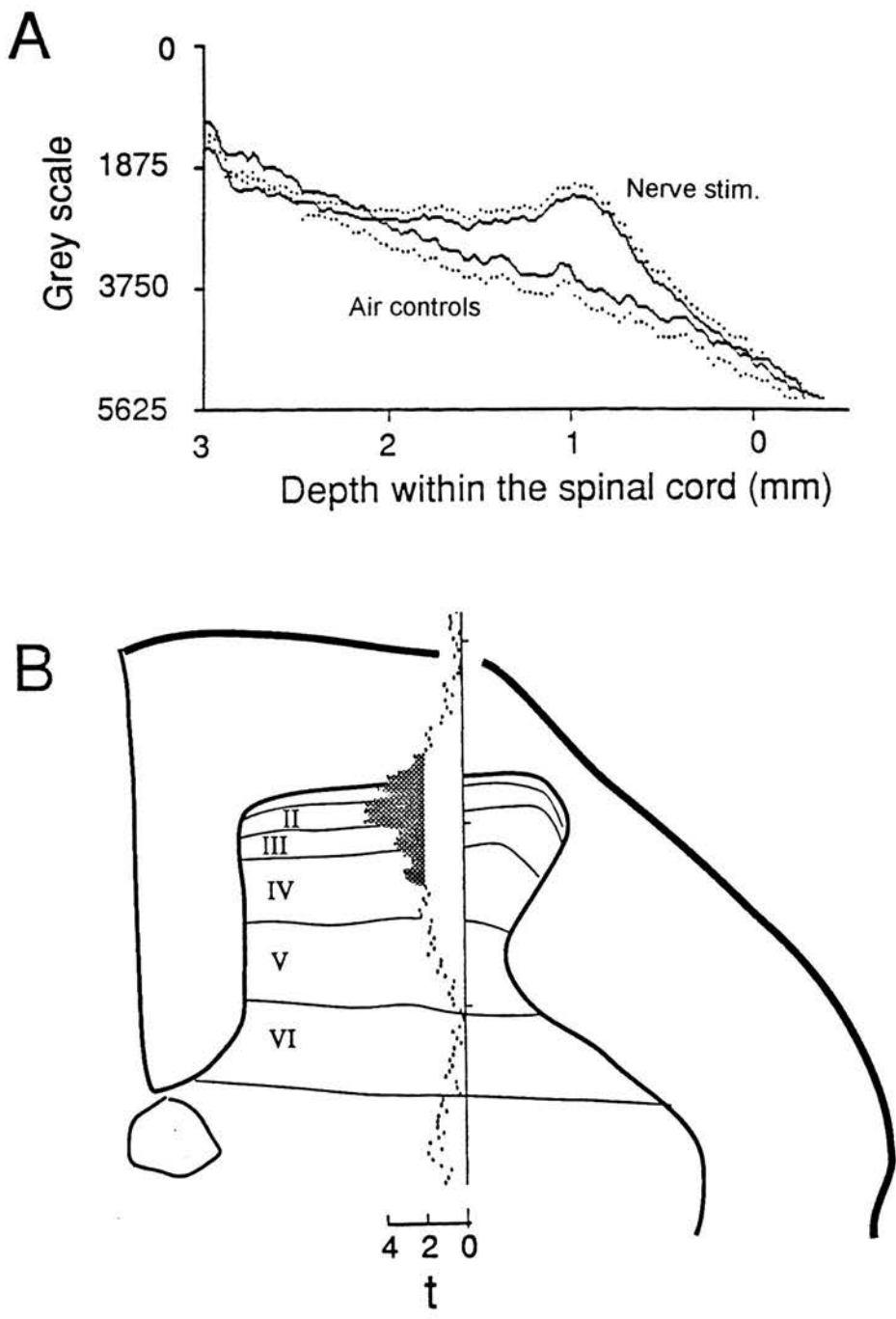


FIGURE 27. Marginally reduced release of immunoreactive substance P during microinjection of neuropeptide Y (NPY) in the superficial dorsal horn. 'Stim. controls', the mean image analysis of a group of microprobes (n=12) inserted 3mm into the dorsal spinal cord during electrical stimulation of the ipsilateral tibial nerve at C-fibre strength but prior to NPY microinjection. 'During NPY', microprobes (n=13) again present in the spinal cord for 20 minutes and during tibial nerve stimulation, but in this case during the slow microinjection of NPY ($10^{-7}/10^{-5}\text{M}$, $0.2\mu\text{m}$) into the adjacent tissue at a depth of 1.1mm. The format of the plot is similar to that in figure 26. A plot of the t-statistics derived from the differences of the means of the two image analyses is shown with respect to distance from the surface of the spinal cord.

FIGURE 27.
EFFECT OF CONCURRENT NPY MICROINJECTION
ON STIMULUS-EVOKED SUBSTANCE P LEVELS.

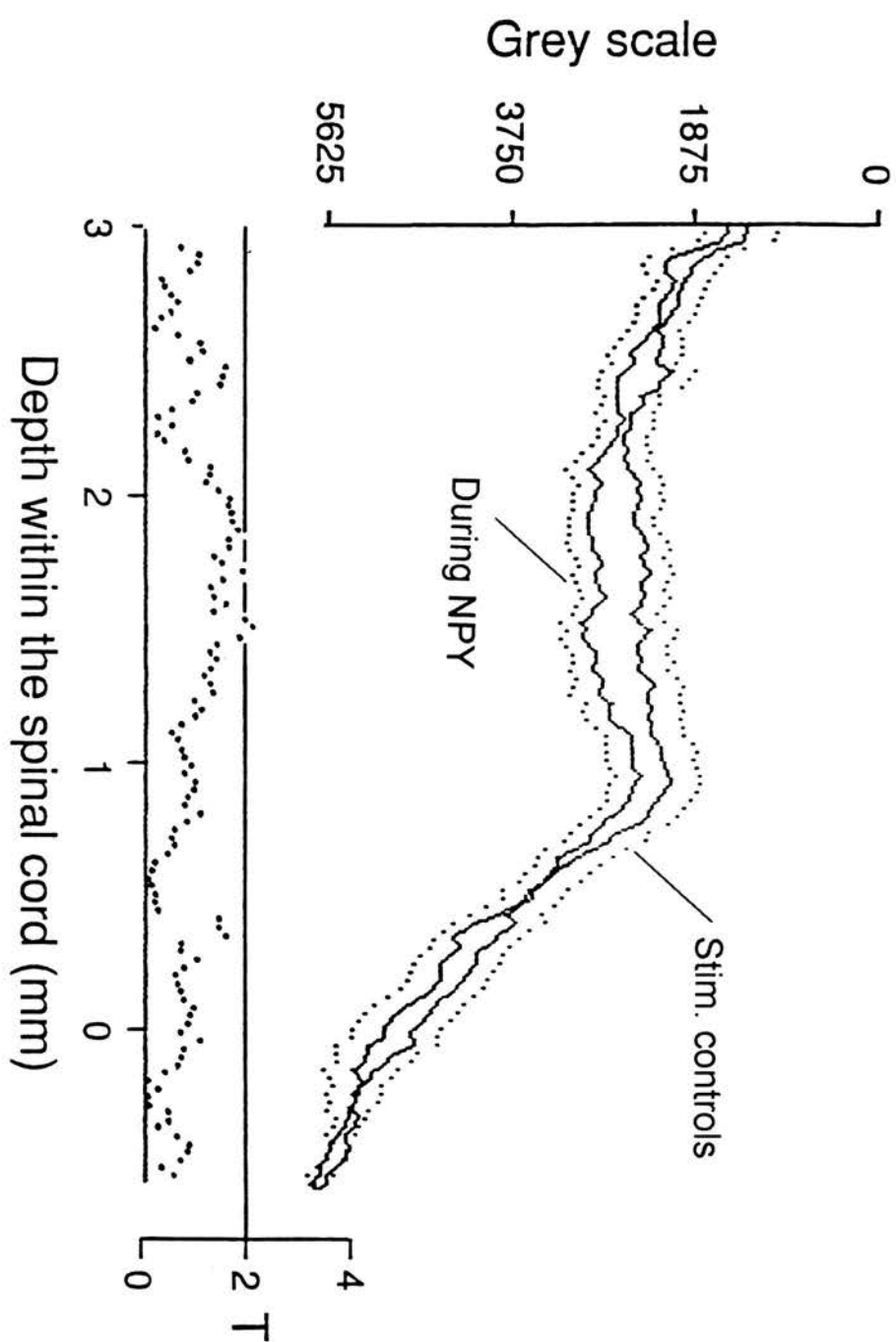


FIGURE 28. Reduced stimulus-evoked release of immunoreactive substance P in the period 0-20 minutes following microinjection of neuropeptide Y (NPY). This figure compares the mean image analysis of microprobes (n=33, 'Stim. controls'), inserted 3mm into the dorsal spinal cord during electrical stimulation of the ipsilateral tibial nerve at C-fibre strength with that of microprobes (n=22, '0-20 min post NPY'), present in the period 0-20 minutes after microinjection of NPY (10^{-5} M). The format of the plot is similar to that in figure 26. A plot of the t-statistics derived from the differences of the means for the two groups of microprobes is also illustrated. The shaded areas on this plot indicate where these differences attain significance ($t > 2$, $P < 0.05$).

FIGURE 28.
EFFECT OF PRIOR NPY MICROINJECTION ON STIMULUS-EVOKED
SUBSTANCE P LEVELS: 0-20 MINUTES.

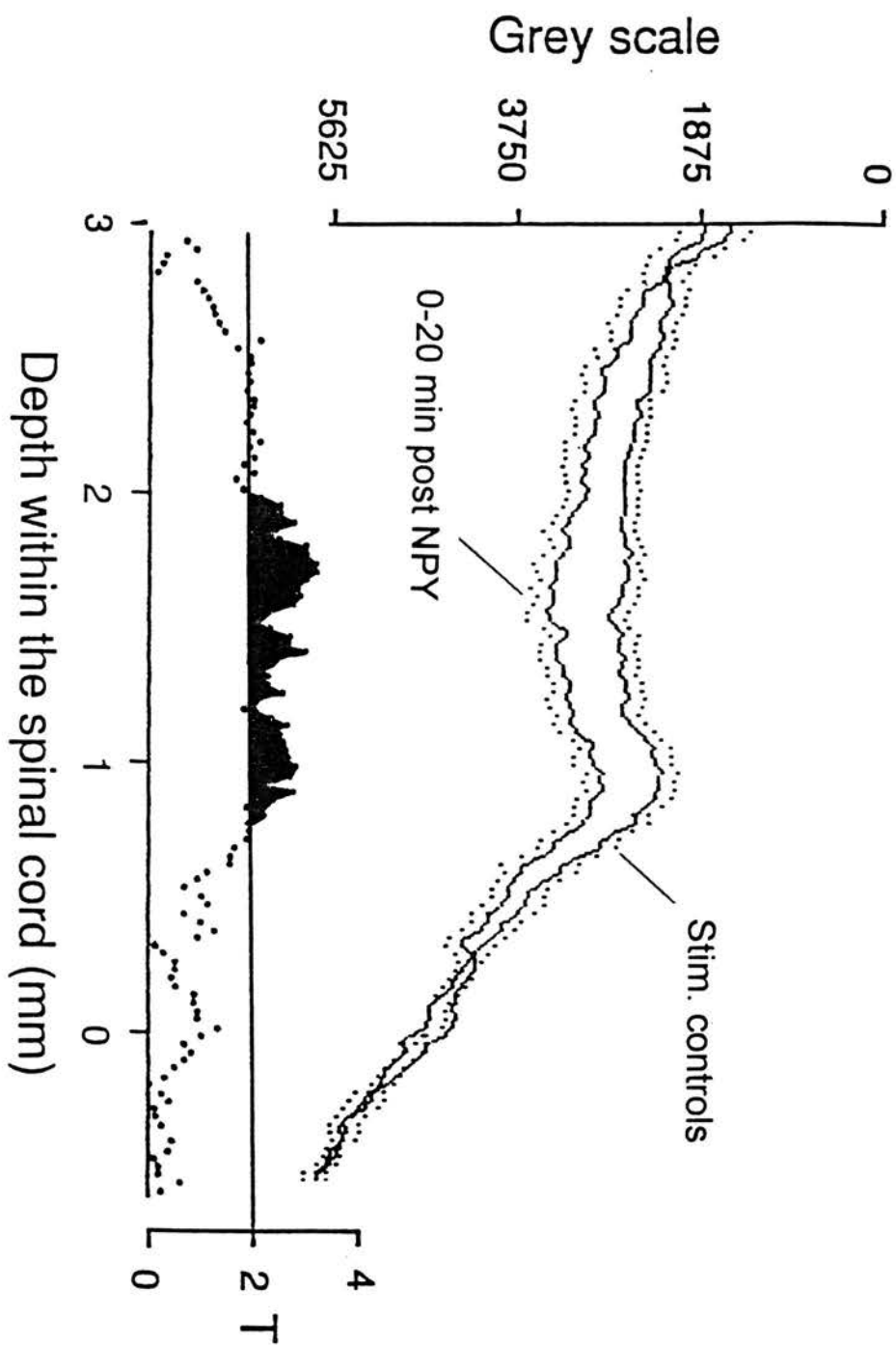


Figure 29. Reduced stimulus-evoked release of immunoreactive substance P in the period 21-40 minutes following microinjection of neuropeptide Y (NPY). This figure compares the mean image analysis of the 'Stim. controls' group as described in figure 28, with that of microprobes (n=20, '21-40 min post NPY'), present in the period 21-40 minutes after microinjection of NPY (10^{-5}M). The format of the plot is similar to that in figure 26. A plot of the t-statistics derived from the differences of the means for the two groups of microprobes is also illustrated. The shaded areas on this plot indicate where these differences attain significance ($t > 2$, $P < 0.05$).

FIGURE 29.
EFFECT OF PRIOR NPY MICROINJECTION ON STIMULUS-EVOKED
SUBSTANCE P LEVELS: 21-40 MINUTES.

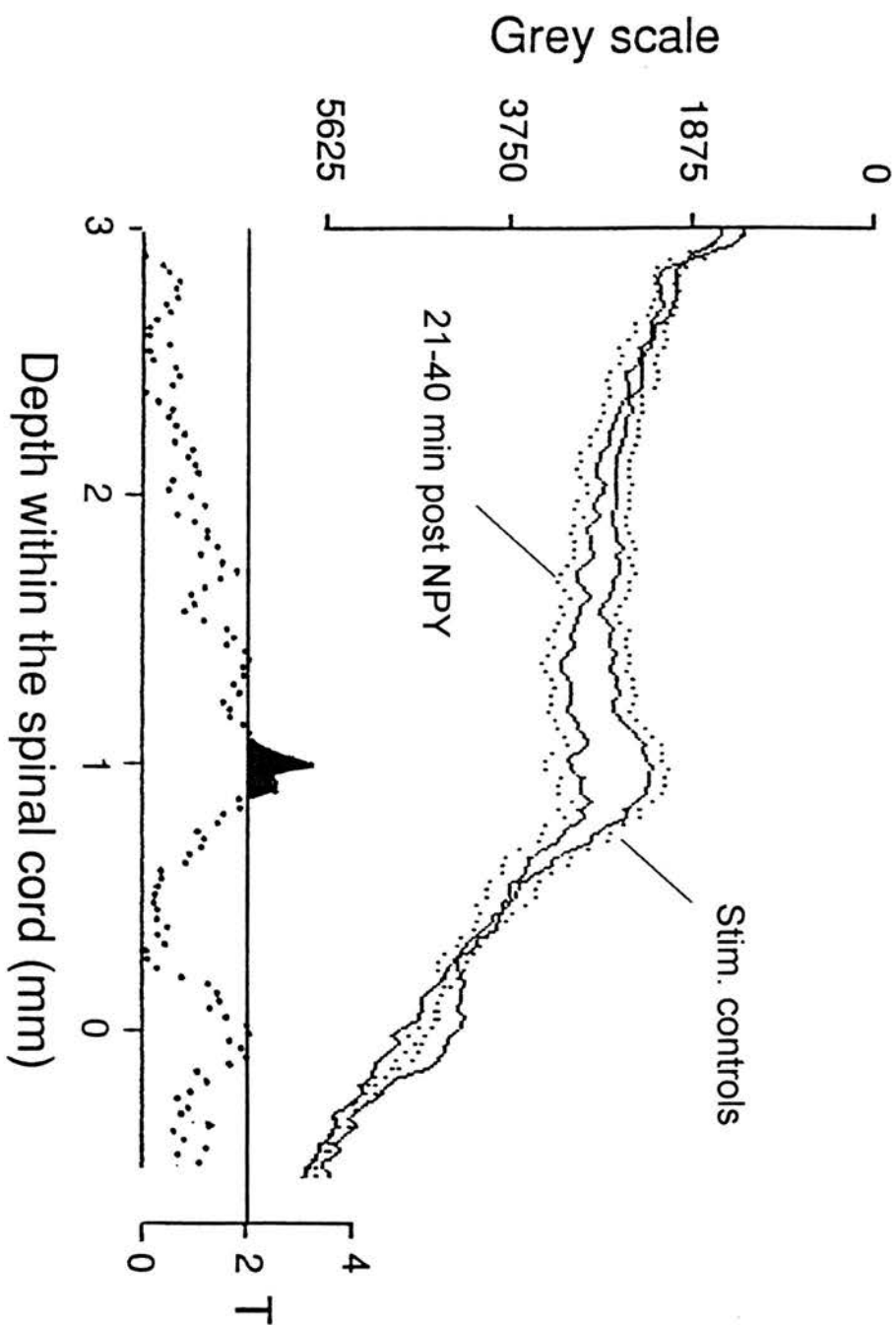


FIGURE 30. Failure of microinjection of phosphate buffered saline (PBS) in the superficial dorsal horn to reduce stimulus-evoked release of immunoreactive substance P. 'Stim. controls', a group of antibody microprobes (n=25) present in the spinal cord for 20 minutes during electrical stimulation of the ipsilateral tibial nerve at C-fibre strength. '0-20 min post PBS', microprobes (n=12) inserted under similar conditions and in the same experiments, but in the period 0-20 minutes after microinjection of PBS into the superficial dorsal horn. The format of the plot is similar to that in figure 26. There are no significant differences between these two mean image analyses.

FIGURE 30.

EFFECT OF PRIOR PBS/AZIDE MICROINJECTION ON STIMULUS-EVOKED
SUBSTANCE P LEVELS: 0-20 MINUTES.

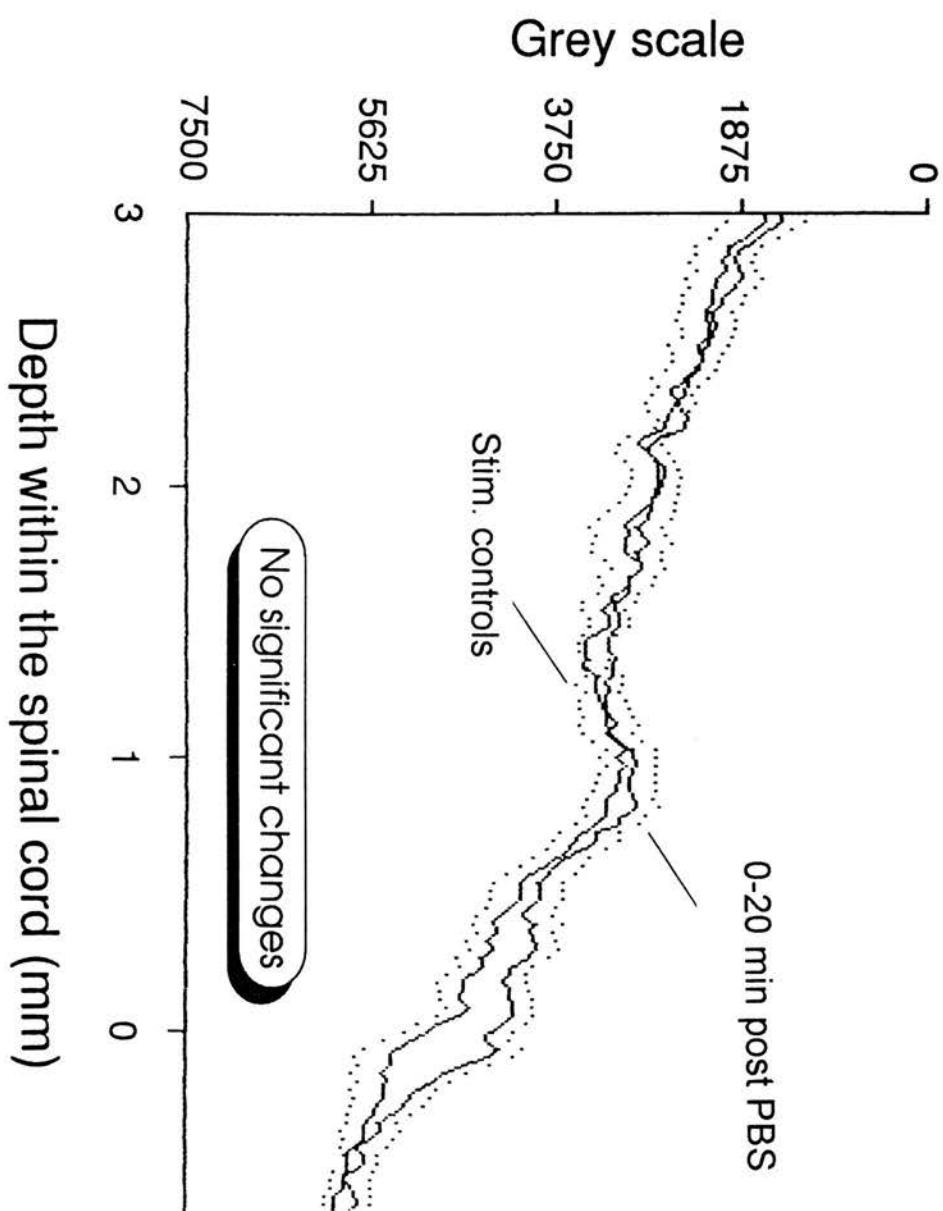
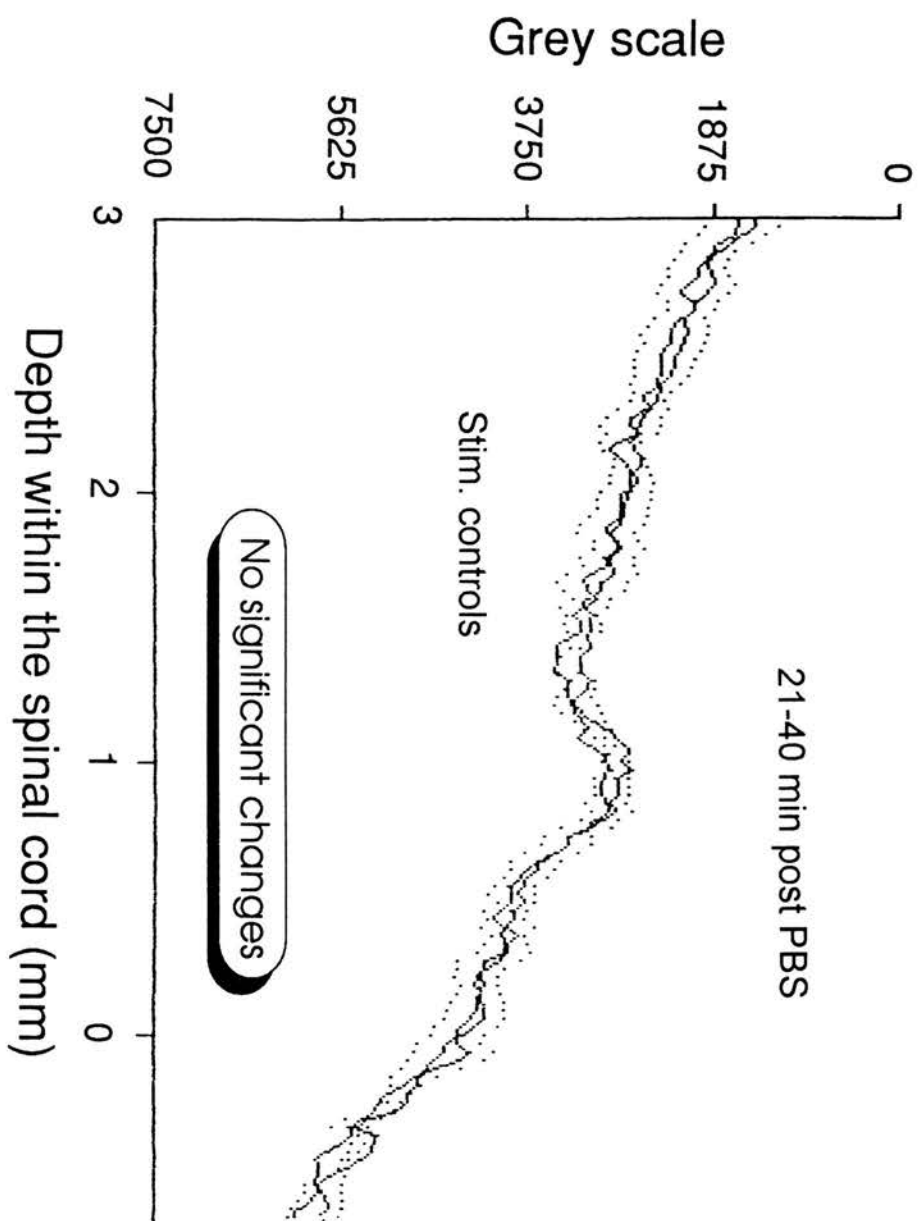


FIGURE 31. Failure of microinjection of phosphate buffered saline (PBS) in the superficial dorsal horn to reduce stimulus-evoked release of immunoreactive substance P. 'Stim. controls', the group of microprobes illustrated in figure 30. '21-40 min post PBS', microprobes (n=7) inserted under similar conditions and in the same experiment but in the period 21-40 minutes after microinjection of PBS into the superficial dorsal horn. The format of the plot is similar to that in figure 26. There are no significant differences between these two mean image analyses.

FIGURE 31.
EFFECT OF PRIOR PBS/AZIDE MICROINJECTION ON STIMULUS-EVOKED
SUBSTANCE P LEVELS: 21-40 MINUTES.



5.7 DISCUSSION.

The results of the experiments in this section have shown that microinjection of 0.2 μ l - 0.6 μ l NPY (10^{-7} M - 10^{-5} M), in the region of the SG of the dorsal horn, reduces the nerve stimulus-evoked release of irSP.

Microinjection of drugs into the spinal cord and interpretation of results.

Interpretation of these results requires consideration of the limitations imposed by microinjection. It was unknown at the time of performing these experiments whether or not NPY would be inactivated quickly or slowly within the spinal cord, and hence the initial experiments microinjected the peptide continuously while microprobes were actually present in the spinal cord. This technique had the disadvantage that the neuropeptide-containing solution was administered at one site only (although relatively close to part of the microprobe shaft) and the time for diffusion away from the site of ejection was restricted when compared with the method of introducing microprobes after microinjection. The findings that greater inhibition of the stimulus-evoked release of irSP was produced by the latter method indicate its suitability for a compound such as NPY. Microinjection probably introduces relatively large amounts of a compound into the spinal cord when compared to the levels achieved by synaptic release, and hence local inactivation processes are likely to be saturated for some time after microinjection. Even with a rapidly degraded compound (such as noradrenaline as in the series of experiments detailed in section 4)

introduction of microprobes after microinjection is probably the method of choice in this type of study. It should, then, be emphasised that the persistence of NPY's effects for up to 40 minutes, as seen in these experiments, may not represent a time course applicable to the effects of NPY in vivo following synaptic release.

It is probable that most of the increases in irSP seen following peripheral nerve stimulation in these and the preceding experiments were due to release from the central terminals of peripheral nociceptors (Duggan and Hendry, 1986, Duggan et al, 1988b). Significant inhibition of irSP release following NPY application occurred over almost the whole of the dorsal grey matter in the first 20 minutes following microinjection but was restricted to laminae I and II in the following 20 minute period. This suggests that the initial 'controls' microprobes, illustrated in **figure 26** were detecting a basal presence of irSP in the deeper laminae of the dorsal horn, that this was increased by nerve stimulation and that microinjection of NPY reduced both basal and stimulus-evoked irSP release. The basal presence of irSP in the deeper laminae of the dorsal horn does, however, contradict the findings of earlier studies performed by members of this same group using the same technique. This issue is one which I will discuss fully in the concluding section of this thesis but it does not, I feel, in any way invalidate the findings of the experiments detailed here.

Presynaptic action of NPY on nerve terminals: histological and physiological studies.

An inhibition, by NPY, of stimulus-evoked irSP release from the central terminals of unmyelinated primary afferent fibres would explain the present results. At the time these results were published the only fine structural study on the relationship between NPY-containing elements and primary afferent pathways was in the lamprey (Bongianni et al, 1990), where varicosities showing immunoreactivity for NPY were found in close apposition to the ascending and descending branches of primary afferent neurones. Since then, Doyle and Maxwell (1993a) have identified axo-axonic contacts in the SG of the cat containing NPY, thus providing an anatomical corroboration for the findings of the present study.

In the periphery, several actions of NPY on nerve terminals have been described. There is evidence that NPY released from sympathetic nerve terminals contacting blood vessels acts as a negative feedback mechanism, controlling not only its own release but also that of noradrenaline (Stjarne and Lundberg, 1986, Wahlestedt et al, 1986). High intensity stimulation of cardiac sympathetic nerves results in prolonged inhibition of the ability of the vagus to slow the heart. This effect, which is mimicked by NPY application, is believed to result from an inhibition of acetylcholine release from vagal endings (Kilborn et al, 1985). Little previous work can be cited on the effects of NPY on tachykinin release, but Guiliani et al (1989) have demonstrated an inhibition, by NPY, of tachykinin release from the peripheral terminals of capsaicin-sensitive nerve fibres in the airways of the guinea-pig.

There is also electrophysiological evidence for presynaptic actions of NPY in the CNS. Studies on cultured dorsal root ganglion, hippocampal and sympathetic neurones (Bleakman et al, 1992, Toth et al, 1993, and Walker et al, 1988) indicate that NPY inhibits calcium influx across the membranes of these cells, possibly via the secondary messenger protein kinase C (Ewald et al, 1988). Inhibition of depolarisation-evoked SP release has also been observed in cultured dorsal root ganglion neurones (Walker et al, 1988) and these two effects may be linked. In the hippocampus of the rat both Colmers et al, (1985,1991) and Hass et al, (1987) demonstrated inhibition of release of an excitatory transmitter, possibly L-glutamate, by NPY (10^{-6} M) added to the perfusate of a slice preparation. It is important to emphasise that these latter effects were obtained in slice preparations which lack a blood supply, since vascular effects need to be considered when any compound is injected into or superfused onto the brain or spinal cord under in vivo conditions. NPY is known to contract blood vessels (Westfall et al, 1988), and so a contribution by such an effect to the present results should not be excluded. It is not, however, known if the small intrinsic vessels of the spinal cord bear NPY receptors.

Possible interactions between NPY and other inhibitory systems.

NPY has been shown to coexist with the classical inhibitory neurotransmitter GABA in neurones of the spinal cord (Rowan et al, 1993) and brain (Aoki, 1989,1990, Jones and Hendry, 1986). As a dense plexus of both NPY-containing fibres (man: Allen et al, 1984, rabbit: Blessing et al, 1987, elasmobranch: Cameron et al, 1990, rat: Chronwell et al, 1985, Henschen et al, 1988, Hunt et al, 1981 cat:

Krukoff, 1987) and noradrenaline-containing fibres (these being largely of supraspinal origin) exist in the dorsal horn of several species (rat: Rajaofetra et al, 1992, Schroder and Skagerberg, 1985, cat: Doyle and Maxwell, 1991a,b,1993b, Lackner, 1980, primate: Westlund et al, 1984), this might be taken as evidence to suggest a coexistence of NPY with catecholamines in descending inhibitory fibres of supraspinal origin. Although NPY does, indeed, coexist with noradrenaline in a subpopulation of neurones in the locus coeruleus, (Everitt et al, 1984, Holets et al, 1988), only 2% of such neurones actually project to the ipsilateral spinal cord as opposed to other areas of the CNS. As the locus coeruleus represents one of the major CNS sites giving rise to the noradrenergic terminals seen in the dorsal spinal cord, this would suggest that coexistence of NPY with noradrenaline in fibres of the dorsal spinal cord is likely to be limited at best.

The effects of NPY in the present experiments could act synergistically to those of noradrenaline in the control of spinal transmission of nociceptive information. NPY is present in intrinsic spinal neurones (Krukoff, 1987, Sasek and Elde, 1985) and release from such neurones might represent a mechanism by which NPY might presynaptically alter release of neurotransmitters from primary afferent terminals ~~s~~ in vivo. As detailed in section 4, intrathecal administration of noradrenaline inhibits spinal reflexes to peripheral noxious stimuli, and microinophoretic administration of noradrenaline in the region of the SG suppresses the transmission of nociceptive information to deeper neurones. My own studies on noradrenaline did not, however, support a presynaptic inhibition of irSP release from primary afferent terminals in the SG underlying

these effects. Noradrenaline has, however, been shown to hyperpolarise neurones of the rat SG in vitro (North and Yoshimura, 1984) and thus a system whereby noradrenaline and NPY, ~~co~~released from terminals in the SG, might interact in the inhibition of primary afferent signal propagation can be envisaged. In such a scheme NPY acts presynaptically on the primary afferent terminal to reduce stimulus-evoked transmitter release, while at the same time noradrenaline reduces the excitability of postsynaptic structures. As previously mentioned, Doyle and Maxwell (1993a) have identified axo-axonic contacts in the SG of the cat containing NPY, thus providing an anatomical corroboration for such a hypothesis.

NPY receptor subtypes.

Inevitably, more than one type of NPY receptor is now recognised (Aicher et al, 1991, Doods and Krause, 1991, Dumont et al, 1992, Potter and McCloskey, 1992). Fuxe et al (1990) have suggested differing functions for the 'high' and 'low' affinity types of NPY receptor. Low affinity NPY (Y1 or postjunctional) receptors are proposed as being localised to subsynaptic areas where high levels of undegraded NPY are encountered. High affinity NPY (Y2 or prejunctional) receptors are proposed to occur remotely from sites of NPY release, their activation relying on diffusion of NPY from those sites; this process has been termed 'volume transmission' (Fuxe and Agnati, 1991), a concept which was discussed much earlier in relation to tachykinin function. A proportion of released NPY molecules may be degraded by peptidases in vivo and one product of this degradation, the fragment NPY (13-36), can be used as a potent and selective agonist for the Y2 receptor (Aicher et al, 1991). This observation

adds further support to the proposition of Fuxe et al that Y2 receptors might mediate the effects of NPY at sites remote from its initial release. High affinity sites for NPY have been identified in the SG of the rat trigeminal nucleus (Nakajima et al, 1986) and hence if the hypothesis of Fuxe et al is correct then a somatotopically diffuse inhibition of SP release may follow synaptic release of NPY. Fortuitously, it is the Y2 receptor which has been implicated in the aforementioned block, by NPY, of calcium currents in cultured dorsal root ganglion neurones (Bleakman et al, 1991).

Conclusion.

The results presented in this section, taken in conjunction with the recent histological findings of Doyle and Maxwell (1993a), strongly support the existence of a presynaptic control of tachykinin release from primary afferent terminals in the superficial dorsal horn mediated by NPY. As discussed, it is most likely that this NPY is derived, in vivo, from intrinsic neurones of the spinal cord itself, but such an inhibitory mechanism might complement the actions of descending inhibitory pathways such as those releasing noradrenaline.

SECTION 6.

GENERAL CONCLUSIONS

My primary intention in this section is not to present a scientific discussion of my findings (this has already been done for the three series of experiments presented in the preceding sections) Rather I would wish to draw together my personal feelings about the results obtained and the overall value of these studies.

The antibody microprobe technique has been applied to studies of neurotransmitter release within several neural tissues. The studies presented here relate more specifically to the investigation of how particular classes of antinociceptive drugs might exert their effects at the level of the spinal cord.

The spinal cord may be viewed as a highly complex system. Synaptic events can be fast or slow, multiple transmitters may be released at many synapses, (influencing the pre- and postsynaptic effects of each other, limiting each others release and possibly even modulating intrasynaptic events). We can, via hybridisation histochemistry, measure the potential production rates of neurotransmitters in vivo and these may be altered as part of physiological control mechanisms. Similarly, cellular production of enzymes and of the membrane substrates for reuptake mechanisms may be up- or down-regulated in particular situations. Multiple receptors exist for almost every identified neurotransmitter and multiple second messenger systems exist within cells to mediate the effects of receptor activation. A multitude of potential neurotransmitters exist and in many cases their functions have not yet been elucidated (even the tachykinins themselves cannot yet be fully classified functionally). The spinal cord is far from static in its physiology, or in popular terms it shows a marked "plasticity". The consequences

(both positive and negative) of this plasticity for clinical pain management regimes are extensive (McQuay and Dickenson, 1990). Into this complexity of mechanisms and interactions we inserted antibody microprobes and tried to make a logical interpretation of our findings. In order to do this we had to make several assumptions regarding the ongoing physiology of the spinal cord.

1) That tachykinins were released in response to peripheral noxious stimulation in a nocispecific manner. Furthermore, that this release was over an anatomically defined area of the spinal cord and was reproducible from one animal to another.

2) That the drugs studied were known to block the transmission of nociceptive information at the level of the spinal cord.

3) That the hypothesis of Jessell and Iversen (1977) could justifiably be applied to these drugs in terms of the known distribution of endogenous neurotransmitters within cat spinal cord.

Studies by our own group and others have been cited throughout this thesis in support of the above points. In relation to point 3) above, however, the work of Doyle and Maxwell (1991a,b,1993a,b) should again be emphasised. This group failed to identify any catecholamine containing axo-axonic contacts in cat spinal dorsal horn, although such contacts staining for neuropeptide Y (NPY) were readily apparent. This work was concurrent with our own and so supports our findings rather than suggesting that the noradrenaline/medetomidine study was misconceived. Similar comments apply to the findings of Cho and Basbaum (1988,1989) on opioid peptide containing contacts in spinal cord.

The initial impetus was to study drugs of veterinary relevance. Opiates and α_2 adrenergic agonists were initially chosen as these represent two classes of drugs used clinically to provide everything from simple analgesia to deep sedation depending on dose. The intention was to determine if these drugs were working, in part, by means of a presynaptic inhibition of nociceptive afferent fibre discharge in the spinal dorsal horn. What can we ultimately conclude from the findings of these studies?

Morphine ,at clinical doses, produces no net inhibition of NKA release in vivo. As a finding in its own right I feel that this is of significance. Such presynaptic inhibition has often been assumed to be one of the main mechanisms of opiate spinal analgesia. Morphine is a very potent analgesic in man and, if correctly administered, equally effective in domestic species, despite the myths and preconceptions which surround its use in species such as the cat where misdosing may cause excitement rather than sedation. As with most neuroactive drugs there are multiple receptors which may be activated by opiate drugs. In the case of morphine most of its analgesic effects will be mediated via mu receptors for which, almost inevitably, subtypes are now being suggested. Morphine also shows significant affinity for delta opioid receptors, and low but detectable binding with the kappa receptor type (Kosterlitz et al, 1985). The effects of morphine at these different receptors are likely to be varied in terms of analgesia (either in terms of which modalities of nociceptive input are blocked, with regard to which part of the transmission pathway is involved, or even in terms of contrasting algesic effects; see Duggan et al, 1981, Fleetwood-Walker

et al, 1988,1990, Hope et al, 1990a, Morton et al, 1987, Onofrio and Yaksh, 1983, Schmauss and Yaksh, 1983, Zhao et al, 1986). Thus we must say that morphine applied systemically has no net effect on primary afferent release of NKA and that it is unlikely that such an effect contributes to the clinical analgesia produced by the drug. What about other opioid/opiate drugs in clinical usage? These have different receptor specificities from morphine and different ranges of effects. Pentazocine, for example is predominantly active at kappa opioid receptors and is used for pain management in various domestic species, although in man it is less popular due to a relatively high incidence of dysphoria associated with its use.

Further studies on the effects of the different opiate analgesics, or maybe more practically of opioid receptor specific ligands, might show different effects on presynaptic inhibition of tachykinin release. The principal problem in the late 1980's, when the morphine study was started, was the relatively limited availability of experimental opioid ligands showing activity in the CNS when administered systemically. In addition to this it was decided that NKA was not the ideal neurotransmitter on which to perform inhibition studies, mainly due to its persistence following release. As Morton et al were at that time studying the effects of opiates on substance P (SP) release (Morton et al, 1990) we ourselves did not perform follow-up experiments on the effects of opioid drugs on tachykinin release. The technique of intra-spinal drug administration was developed in the subsequent study on adrenergic agonists.

Noradrenaline/Medetomidine The effects of these compounds (administered intraspinally) on stimulus-evoked SP release were studied. Noradrenaline, the classical example of an endogenous α_2 adrenergic receptor agonist, did not reduce stimulus evoked SP release. The technique of spinal drug administration was a new one to our group and rather than simply increasing the concentration of noradrenaline used (which would have increased α_1 receptor activation) we opted to use the new imidazoline compound medetomidine which has a much higher α_2 selectivity. This was also without effect. The results of this study are perhaps the most controversial of those presented here. Noradrenaline has classically been associated with descending and segmental inhibitory pathways in the spinal cord and its actions have (as with opiate drugs) often been attributed to pre-synaptic inhibition at primary afferent terminals. The lack of effect of medetomidine and the findings of Doyle and Maxwell (1991a,b, 1993a,b), do however strengthen our case. SP is a much better choice of compound for these studies than NKA, being short-lived in vivo and more restricted in its distribution following release. A more interesting result would have been an inhibitory effect evident with medetomidine but not with noradrenaline. Such an effect would have at that time been attributed either to activation of imidazoline binding sites by medetomidine or an antagonistic effect mediated by α_1 receptors activated (significantly) by noradrenaline only. Further studies utilising available antagonists would then have been of great interest; regrettably the necessity for these did not arise.

Neuropeptide Y Studies on NPY ran in parallel with the noradrenaline studies. The potential for an interaction, in modulating primary afferent transmission, between NPY and noradrenaline have already been discussed in section 5. Microinjections of small volumes of NPY at concentrations of 10^{-7} to 10^{-5} M did inhibit the noxious stimulus evoked release of SP. To date this is the only compound which has been shown, with the antibody microprobe technique, to inhibit tachykinin release from primary afferent terminals. The support of our findings by the concurrent anatomical studies of Doyle and Maxwell (1993a) has already been emphasised.

What have these studies contributed to the field of neuroscience? The morphine and $\alpha 2$ adrenoceptor agonist studies have, at least, indicated that these drugs do not work by the presynaptic mechanism often attributed to them (at least in terms of primary afferent pathways utilising tachykinin peptides). This indicates a possible area for the development of new analgesic drugs ie. compounds which do target these sites of neurotransmitter release. NPY appears to do just this, but NPY is a neuropeptide widely distributed in the central nervous system (CNS), and difficulties might arise with the use of drugs active at its receptors in a clinical context. Systemic administration of such drugs might have diverse effects in the whole animal, some of these effects being detrimental to the animals well-being. Morphine, however, has become popular and is effective as a systemically administered analgesic drug despite having a similar 'whole-nervous system' effect. In the case of morphine the clinical advantages of its use outweigh the

disadvantages (respiratory depression, tolerance, addiction, dysphoria etc). Drugs active at NPY receptors might prove to have a similar positive balance of therapeutic value. The NPY study is also of great value to our research group, however, providing evidence that antibody microprobes can be used to detect quantitative changes in immunoreactive SP release over a period of time.

Further studies. Incorporated in the end of this volume are reprints of papers published from the results of the antibody microprobe studies with which I have been involved during my period of study in Edinburgh. Several different compounds have been successfully measured in CNS structures, including NKA, SP, beta-endorphin and galanin, and studies have been performed in both the cat and rat. The antibody microprobe technique can be applied to the detection of many peptides but it must be noted that not all studies are successful. To date studies on endothelin and dynorphin release have failed to yield consistent data, and a study on the effects of intraspinal serotonin administration on SP release also presented technical difficulties.

Antibody microprobes, in their present form, are best suited to the detection of peptides in neural tissue on a simple yes/no basis. It is not possible at the present time to make them truly quantitative. Gross changes in peptide distribution following drug administration are readily detected (eg. the effects of calcitonin gene-related peptide (CGRP)/peptidase inhibitors on the pattern of stimulus-evoked SP release), but more subtle changes may be beyond the present limit of antibody microprobe sensitivity. Modifications

of the technique may be possible in the future and I would divide such changes into three areas:

1) Improvement of sensitivity. Maximisation of density and affinity of ligand binding sites on microprobe surfaces. This may involve the production of higher affinity antisera against the peptides being studied or alternatively changes to the way in which antibody molecules are chemically bound to the microprobes.

Comments have already been made about the subtle differences in the pattern of stimulus-evoked SP release in the spinal cord between early (Duggan and Hendry, 1986, Duggan et al, 1988b) and more recent antibody microprobe studies (notably Duggan et al, 1991a, see also section 5), and these may simply be due to improved antibody microprobe sensitivity. Thus the original antibody microprobe papers of Duggan and co-workers (1986,1988b) revealed a stimulus-evoked pattern of irSP release confined to the superficial laminae of the dorsal horn, and a basal presence which was not only an inconsistent finding but which was in low levels when present and which was certainly not detectable in the deeper laminae of the dorsal horn. The existence of a basal presence of irSP has never been re-evaluated per se, but subtle changes in the antibody microprobe technique over the last five years may account for a basal presence of irSP now being detectable, as illustrated in section 5.

2)Improvement of image analysis systems. Iodinated markers for unoccupied antibody binding sites do provide a high resolution means of imaging the distribution of peptide on microprobe surfaces. The main drawback of this technique is the delay (in some cases of weeks)

between the experiment and the visualisation of results. The development of fluorescent, rather than radioactive, markers may allow a more "instant" analysis of peptide distribution on microprobes. Taking this one step further it may in the future be possible to perform such analysis in situ ie. with the peptide binding itself producing fluorescence and the light produced being measured optically from within the microprobe barrel.

3) Application of the technique to different media and sites eg. using antibodies bound to planar surfaces as opposed to conical forms. Such modification (with suitable alterations in image analysis technique) might allow the visualisation of peptide release from, for example, meninges or structures of the gastrointestinal tract. The basic principles of the antibody microprobe technique might also be applied to cell culture media in order to monitor the peptide production of growing cells.

These are changes which might be pursued in the antibody technique itself. Different types of study would now also be of interest with a view to extending the results obtained over the last few years. It is clear that the afferent terminal is by no means the only site at which analgesia can be produced. Even confining studies to the first synapse, intra-synaptic events (again as in the case of the CGRP/peptidase inhibitor studies) may be further manipulated with the aim of producing analgesia. At the post-synaptic membrane the many receptor subtypes may all mediate different effects. Receptor specific antagonism as opposed to blockade of neurotransmitter release may prove to be a more viable means of producing a defined

analgesia without other functional deficits. The blockade of specific second messenger systems may be of equal value, but before this can be assessed the actual functions of these receptor/messenger subtypes must be evaluated. Such studies are currently being undertaken by various groups (Fleetwood-Walker et al, 1993, Parker et al, 1993, Picard et al, 1993, Thompson et al, 1993, Wienrich et al, 1989).

Whole animal studies are also of interest, particularly if they can be directly applied to clinical case material. I would propose that it should now be possible (utilising some of the techniques used in the studies presented here) to develop a biochemical assay for "pain" in domestic species. Pain is of course a perceptual event, not readily quantifiable in domestic species. What would really be measured would be maintained nociceptive input from either somatic or visceral structures to the CNS. Plasma levels of NKA may reflect such ongoing input, and their evaluation by radioimmunoassay may provide a useful diagnostic/prognostic tool in veterinary and human medicine. Although SP has been measured in this fashion in human plasma, cerebrospinal fluid, saliva and articular fluid (Marshall et al, 1990, Parris et al, 1990, Tamsen et al, 1982) such work has provided no clear correlation with pain perception and studies on NKA are curiously limited. Measurement of dynorphin levels in a similar fashion (perhaps in cerebrospinal fluid) may equally be of value in the assessment of spinal cord injuries.

With the advent of more refined analytical and experimental techniques the apparent contributions that the tachykinin peptides and their receptors make to spinal cord processing continue to increase. As our understanding of the whole field of neuroscience becomes more complete, it is clear that many, many opportunities for the development of novel analgesics must exist but at the same time our overall understanding of how the spinal cord actually functions is in some ways getting more confused. The true picture of spinal cord processing is a very large and very complex puzzle; we are still taking the pieces out of the box. I hope that the work presented in this thesis has helped to shape a couple of those pieces.

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Evidence for Localized Release of Substance P Within Rat Spinal Cord Evoked by Physiological and Electrical Stimuli

C. W. LANG and P. J. HOPE

Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK

Abstract—Antibodies immobilized onto the outer surface of glass microelectrodes were used to measure and localize substance P (SP) release in the spinal cords of anaesthetized rats. Utilizing a C-terminally directed antibody, significant levels of SP were not found in the lumbar spinal cord in the absence of peripheral noxious stimulation. Following noxious heating or pinch of the ipsilateral hind paw or electrical stimulation of the ipsilateral tibial nerve at C-fibre strength, significant amounts of released SP were detected. This noxious stimulus-evoked release of SP was primarily in the region of the substantia gelatinosa. In conclusion, the antibody microprobe technique can be employed to focally detect the release of neuropeptide *in vivo*, even in structures as small as rat spinal cord. The technique reveals that SP release in the rat follows broadly the same pattern as that previously reported in the cat.

Introduction

Substance P (SP) is known to be involved in the spinal processing and transmission of nociceptive afferent inputs.^{1–6} Many of the studies seeking to elucidate the functions of SP, whether behavioural, histological, pharmacological or physiological in nature, employ the rat as an experimental model. To date, the release of SP within rat spinal cord *in*

vivo has been assessed by perfusion techniques,^{7–9} but such techniques suffer from poor spatial resolution. The smaller amounts of peptide contained in rat spinal cords, as compared to those of larger mammalian species such as the cat or rabbit, may also present a problem in the *in vivo* measurement of peptide release. Techniques involving the collection of peptide in a perfusate will dilute it to even smaller concentrations, and the possibility exists that release will be undetected following mild stimuli. Thus, to date, both *in vivo* and *in vitro* studies on rat spinal cord have described only 40 mM potassium or 10 M capsaicin application, or repeated

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Correspondence to: C. W. Lang, Department of Physiology, Medical School, University of Bristol, Bristol BS8 1TD, UK.

flexion of a polyarthritic limb as being adequate stimuli to evoke SP release.

In recent years the antibody microprobe technique of Duggan et al.^{10,11} has been used to study the distribution of release of various neuropeptides in the cat spinal cord, and also to determine the adequate stimuli required to evoke that release.¹² Antibody microprobes have several advantages over other methods of *in vivo* neuropeptide measurement: 1) they are physically much smaller than push-pull cannulae or microdialysis tubing, and cause little apparent physiological or anatomical disruption in their utilization; 2) they are capable of detecting even very small amounts of peptide, estimated as low as 10^{-17} moles peptide/microprobe; 3) they offer a much better spatial resolution than other methods (in the order of 100 μ m), and are consequentially better suited for the localization of peptide release within small structures or within discrete areas such as the substantia gelatinosa of the rat.

Antibody microprobes showed that immunoreactive SP (irSP) is released in the cat lumbar spinal cord, *in vivo*, in response to noxious mechanical and thermal stimulation of the hind paws and also in response to electrical stimulation of the tibial nerves at C-fibre strength.¹² Whichever stimulus type was employed, the evoked release of irSP was predominantly centered in the region of the substantia gelatinosa, with a smaller amount of release in the region of lamina V of the grey matter. Basal presence of irSP in the absence of peripheral noxious stimulation was not a consistent finding, but when present, basal irSP was detected only in the region of the substantia gelatinosa, and in much smaller amounts than that evoked by subsequent noxious stimulation.

The rat is, as previously described, a much more common model for the study of SP function and nociceptive processing than the cat: in recent years, for example, the development of new models for neuropathic pain have all involved the rat.¹³⁻¹⁵ The aim of the current study was to determine the distribution of stimulus evoked irSP release in rat spinal cord *in vivo* in comparison to that previously described in the cat, and additionally to examine whether or not the adequate stimuli for irSP release employed in cat studies could also be applied to the rat.

Methods

Microprobe preparation

Antibody microprobes were prepared as previously described.^{10,11} Briefly, fine glass micropipettes were heat sealed at both ends and incubated in a 10% solution of aminopropyltriethoxysilane in toluene. This produced a siloxane polymer on the outer surface of the glass to which protein A (Sigma) was immobilized with glutaraldehyde coupling. The protein A was then utilized to bind immunoglobulins present in a polyclonal antiserum raised against the C-terminal of SP (Peninsula Laboratories). Manufacturers data for this antiserum stated no cross-reactivity for neurokinin A, neurokinin B or neuropeptide K. The sensitivity of the prepared antibody microprobes was assessed by incubating a small number of the probes in 10^{-7} M SP *in vitro* at 37°C for 30 min, prior to subsequent incubation in a solution of Bolton-Hunter ¹²⁵I-labelled SP (Amersham) diluted to 2000 cpm 1^{-1} . This *in vitro* incubation in unlabelled SP was, in all tests, sufficient to reduce subsequent ¹²⁵I binding by at least 50%. All incubations, whether during probe preparation, *in vitro* testing, or following *in vivo* use were carried out in 51 capillaries of the relevant solution, with the exception of antibody to protein A binding which used 12.51 capillaries. Incubations in these capillaries were, except for the unlabelled SP *in vitro* mentioned above, for 24–48 h at 4°C.

Animal preparation

Experiments were performed on 13 rats (male and female, 220–270 g). Anaesthesia was induced with sodium thiopentone (120 mg kg^{-1} , 45 mg ml^{-1} i.p.) and maintained with periodic top-up doses of the same drug (15 mg kg^{-1} , 15 mg ml^{-1} i.p.). Blood pressure was monitored via carotid cannulation, and the trachea was also cannulated. The lower thoracic/upper lumbar spinal cord was exposed by dorsal laminectomy and a solution of 3% agar in sterile saline was injected under the rostral end of the dissection and caudally to cover the exposed surface of the cord. In all experiments the tibial nerve in one hindlimb was exposed to allow electrical stimulation.

To allow microprobe insertion an opening was

made in the agar covering the cord to form a pool which was then irrigated with warmed, sterile Ringer solution. Within this pool the dura mater was cut longitudinally and reflected laterally. Pairs of microprobes, placed 3 mm apart rostrocaudally, were inserted 2.25 mm into the spinal cord with a stepping motor micromanipulator. By filling the first microprobes with saline, or by using a silver ball electrode placed on the cord surface, electrophysiological recordings were made to determine the best area for microprobe insertion with regard to the field potentials evoked by stimulation of the ipsilateral hind limb. The last microprobes inserted into the spinal cord were used to ionophorese pontamine sky blue dye to allow subsequent histological verification of microprobe placement.

Peripheral stimulation was by either electrical stimulation of the ipsilateral tibial nerve at C-fibre strength (10–20 V, 20 Hz, 0.5 ms pulse width) or by heating, in water at 48°C, or noxious pinching of the ipsilateral hindpaw. Noxious heating of the paw involved placing the paw in a beaker of water preheated to the desired temperature and insulated to prevent loss of heat during the period of the test. Noxious pinch was by the application of a single crocodile clip dorso-ventrally across all digits of the ipsilateral paw. Noxious stimuli were, when applied, of 2 min duration, and followed placement of microprobes within the spinal cord. Microprobes were left in situ for 8 min following cessation of noxious stimulation or, where no peripheral stimulus was applied, for 10 min.

Following removal from the spinal cord microprobes were washed for 15 min in cold phosphate-buffered saline (PBS) containing 0.1% Tween and incubated for 24 h at 4°C in ^{125}I Bolton Hunter labelled SP. Probes were then washed for a further 15 min in cold PBS and the tips were broken off and fixed to a sheet of cartridge paper. This sheet was placed in an X-ray film cassette loaded with Kodak, NMB monoemulsion film.

X-ray film images of antibody microprobes were analysed with a computer based image analysis system employing an Image Technology PC frame grabber board operating in a DCS 286e (AT) computer. A CCD camera scanned each image, and as previously described,¹⁶ following background subtraction, a transverse integration of optical density was performed for each microprobe at defined

intervals. With the resolution of the analysis system used, this corresponded to a 10 m interval between each integration. The resultant plot of optical density, or grey scale, for each individual probe image was then stored as a file on hard disk along with 37 coded values representing the experimental parameters applying to that particular microprobe. Any reduction in grey scale value along the length of the microprobe equates to binding of endogenous irSP and subsequent failure to bind the otherwise expected amount of ^{125}I -labelled tracer. An analysis program was used to group together individual plots for microprobes meeting stated experimental criteria, and produce for each group a plot of mean grey scale value with respect to depth within the spinal cord. For the purposes of further analysis (standard error of means and statistical significance of differences between means), integration points were grouped in threes, bringing the spatial resolution of the analysis to 30 m. Mean image analyses could then be compared, both visually, and statistically by the Student's *t*-test. This method of analysis thus samples release at many sites within the spinal cord, with a resolution of 30 sites mm^{-1} .

Results

A total of 94 microprobes coated with antibodies to SP were inserted into the spinal cord and a further 48 microprobes were used for concurrent in vitro testing as described.

Controls

35 microprobes were inserted 2.25 mm into the spinal cord for a period of 10 min and in the absence of any prior or concurrent noxious peripheral stimulation. In the spinal lumbar enlargement this places the tips of the microprobes just below or at the base of the ventral grey matter. The Student's *t*-test was used to compare the mean grey scale image of the control group with one derived from in vitro microprobes not exposed to SP prior to incubation in labelling peptide. No statistically significant differences were seen at any point in the dorsal spinal cord, thus a basal presence of irSP was not detected.

Electrical stimulation of ipsilateral tibial nerve

Figure 1A shows the mean image analysis of a group of 13 microprobes inserted into the spinal cord for 10 min with a concurrent 2 min period of electrical stimulation of the ipsilateral tibial nerve above C-fibre threshold (10–20 V, 20 Hz, 0.5 ms pulse width). The mean image of the control group of 35 microprobes, inserted for 10 min in the absence of any peripheral stimulation is also illustrated. Figure 1B shows a plot of the t-statistics for the differences between these 2 image analyses with a diagram of cord structure for reference. The shaded area on this t-plot represents the sites of significant differences ($t > 2$, $p < 0.05$) between the 2 mean images. Electrical stimulation of the ipsilateral tibial nerve in this manner resulted in statistically increased amounts of irSP being detected over an area approximately 0.5 mm wide, extending from the cord surface to within the dorsal horn of the spinal grey matter, with the area of maximal release being centered on the superficial laminae of

the dorsal horn. A much smaller zone of significant release was seen approximately 0.75 mm below cord surface. This smaller, deep zone of release was not evident when noxious mechanical or noxious thermal stimuli were employed.

Noxious mechanical stimulation

Figure 2A shows the mean image analysis of a group of 15 microprobes inserted 2.25 mm into the spinal cord for 10 min with a concurrent 2 min period of noxious pinching of the ipsilateral hind paw by means of application of a crocodile clip across the phalanges, placed dorso-ventrally. As in Figure 1A, the mean image analysis of the control group is also illustrated. Figure 2B shows a t-plot for the significance of the differences between these 2 groups. The stimulus-evoked release of irSP in the dorsal spinal cord is similar in spread to that seen with tibial nerve stimulation, although the small, deeper zone of release is absent.

At the end of each experiment it was observed that repeated application of the alligator clip (to a

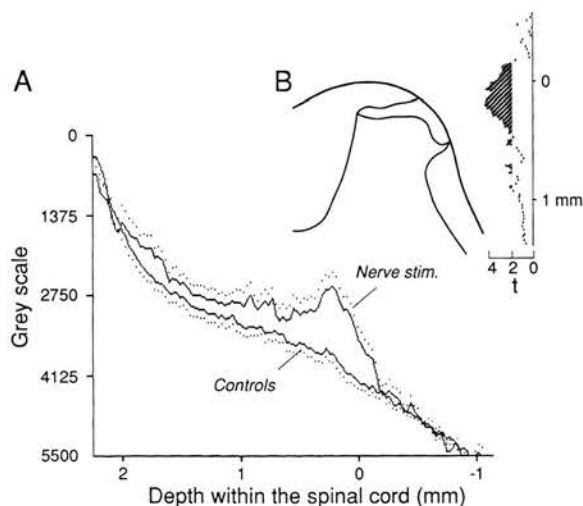


Fig. 1 Ipsilateral tibial nerve stimulation (above C-fibre threshold) resulted in detection of irSP in the superficial dorsal horn. (A) The mean image analyses of controls (no applied peripheral stimulation, 10 min microprobe insertion, $n = 35$) and nerve stimulus microprobes (nerve stim.) (20 Hz, 10–20 V, 0.5 ms pulse width, applied for 2 min out of 10 min microprobe insertion period, $n = 13$) are shown. (B) The significant increase in detected irSP following electrical stimulation of the ipsilateral tibial nerve is confirmed in the plot of the t-test values derived from the 2 groups shown in Figure 1A. The shaded area represents points where the t-value exceeds 2.0 ($p < 0.05$), indicating detection of increased irSP at this level of significance. This plot of t-values is related to a diagram of rat lumbar spinal cord.

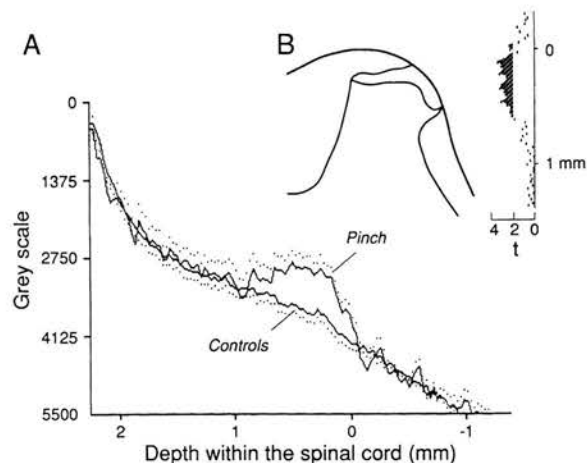


Fig. 2 Noxious mechanical stimulation of the ipsilateral hindpaw resulted in detection of irSP in the superficial dorsal horn. (A) The mean image analyses of controls (no applied peripheral stimulation, 10 min microprobe insertion, $n = 35$) and pinch (crocodile clip applied to ipsilateral hindpaw for 2 min out of 10 min microprobe insertion period, $n = 15$) antibody microprobe groups are illustrated. (B) The significant increase in detected irSP following pinching of the ipsilateral hindpaw is confirmed in the plot of the t-test values derived from the 2 groups shown in Figure 1A. The shaded area represents points where the t-value exceeds 2.0 ($p < 0.05$), indicating detection of increased irSP at this level of significance. This plot of t-values is related to a diagram of rat lumbar spinal cord.

maximum of 8 min total) had not caused obvious bruising or oedema in the soft tissues of the paw.

Noxious thermal stimulation

The mean image analysis of 29 microprobes inserted 2.25 mm into the spinal cord for 10 min, during which time the ipsilateral hind paw was placed in water at 48°C for 2 min, together with the control group mean image analysis is illustrated in Figure 3A. A plot of *t*-statistics derived from the differences between these 2 groups is shown in Figure 3B. Noxious thermal stimulation evoked significant irSP release over a similar area in the dorsal horn to that seen with the other types of noxious peripheral stimulation. The small deep zone of release seen following tibial nerve stimulation was, however, absent. This stimulus-evoked release of irSP following noxious thermal stimulation was less marked than that obtained with the other types of stimulus, but it was more obviously centered within the superficial dorsal horn.

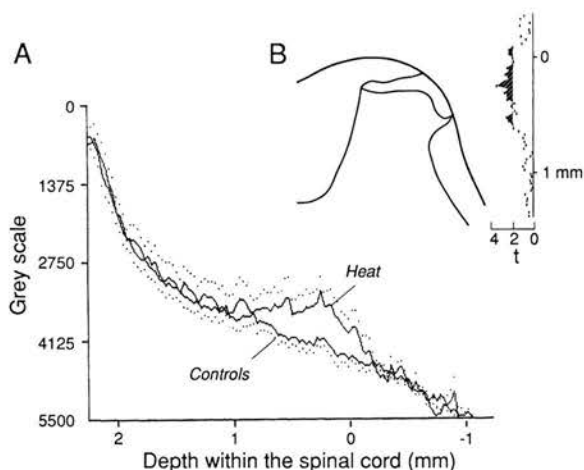


Fig. 3 Noxious thermal stimulation of the ipsilateral hindpaw resulted in detection of irSP in the superficial dorsal horn. (A) The mean image analyses of controls (no applied peripheral stimulation, 10 min microprobe insertion, $n = 35$) and heat (immersion of ipsilateral hindpaw in water at 48°C, for 2 min out of 10 min microprobe insertion period, $n = 29$) antibody microprobe groups are illustrated. (B) The significant increase in detected irSP following heating of the ipsilateral hindpaw is confirmed in the plot of the *t*-test values derived from the 2 groups shown in Figure 1A. The shaded area represents points where the *t*-value exceeds 2.0 ($p < 0.05$), indicating detection of increased irSP at this level of significance. This plot of *t*-values is related to a diagram of rat lumbar spinal cord.

Discussion

The detection of noxious stimulus-evoked release of irSP in the superficial dorsal horn is consistent with the hypothesis that, in the rat, SP is released from the central terminals of nociceptive afferent fibres.^{17–21} The major location of such terminals derived from the skin of the rat is the superficial dorsal horn; laminae I–II of the dorsal grey matter.²² The absence of basal levels of irSP in the dorsal spinal cord in the present study is in agreement with earlier work in cat spinal cord where either no irSP or very small basal amounts were found in the area of the substantia gelatinosa in the absence of prior noxious stimulation.^{12,23,24} Although the rat spinal cord is anatomically much smaller than that of the cat, and hence the absolute amounts of a given neuropeptide, whether basal or stimulus-evoked, will also be smaller by comparison, concentrations at sites of release are probably similar. The small, discrete zones of irSP detection in the deep dorsal horn seen following nerve stimulation (Fig. 1B) indicate that microprobes are sensitive enough to detect such limited release even over a very small area. Given this high sensitivity (each individual antibody microprobe is capable of detecting as little as an estimated 10^{-17} moles of peptide),¹⁰ it is unlikely that a basal release of irSP in the rat superficial dorsal horn went undetected in the present study.

In the rat, as in the cat, noxious stimulus evoked release of irSP was centered on the superficial dorsal horn which includes the substantia gelatinosa (laminae I and II) of the spinal grey matter. The area of significant increases in irSP detection seen following peripheral stimulation in the present study (Figs 1B, 2B and 3B) appears at first to be more diffuse than the distribution of irSP revealed in immunocytochemical studies.^{17–21,25} There are several possible explanations for this. SP, and also the other members of the tachykinin neuropeptide family, are probably not subject to any rapid reuptake mechanisms but rather are inactivated by peptidase metabolism. In plasma or cerebrospinal fluid, *in vitro*, SP has a half life in the order of 2 min, which would be long enough if applied to *in vivo* conditions to allow some spread away from the initial sites of release.²⁶ This may explain our finding of irSP in the dorsal columns. In cat studies such

dorsal spread into the white matter has been observed with antibody microprobes, resulting in a typical zone of stimulus-evoked irSP detection approximately 1 mm wide, although like the present findings in the rat, maximal in and centered on the superficial dorsal horn. The typical zone of stimulus evoked irSP detection reported in this study is approximately 0.5 mm wide, which given the different sizes of the spinal cords of the 2 species is comparable to the 1 mm wide zone seen in the cat. The increases in irSP following noxious thermal stimulation (Fig. 3B), whilst lower than those seen following pinch (Fig. 2B) or nerve stimulation (Fig. 1B) do appear to be better demarcated in terms of localization within the spinal cord. This would support the hypothesis that the relatively broad zone of stimulus-evoked irSP detection seen in all cases in the rat might be due simply to spread away from sites of release as a consequence of the limit of the rate of peptide breakdown *in vivo*. Furthermore, the distance of the substantia gelatinosa from the dorsal surface of the spinal cord varies more greatly in the rat than in the cat with respect to laterality. Pooling of microprobes inserted at varying distances from the cord midline may then result in a greater 'blurring' of localization in the rat, making the resultant zone of irSP detection seem disproportionately wide. Small errors in probe placement, or breathing movements transferred to the cord will also be relatively more significant when studying smaller structures, thus adding to the above effect. Indeed individual microprobe images, as opposed to the pooled mean images, did frequently show a much more restricted zone of irSP detection, although this was still centred in the superficial dorsal horn.

Previous studies of SP release in the rat *in vivo* have employed models of inflammatory disease to evoke release, such as flexion of an arthritic joint or pinching of a formalin injected paw.^{8,27} We have shown in the present study that noxious stimuli applied to undamaged tissues; pinch and heat to normal paw, and also electrical stimulation at C-fibre strength of peripheral nerve, were adequate in evoking irSP release in the rat. There is one important difference, however, between the present results and those previously obtained in the cat. In the cat immersion of the hind paw in water at 48°C was ineffective in producing significant increases in

detected irSP; water temperatures of 50 or 52°C were required. The rat foot is different in structure from that of the cat. The skin is thinner and there is less hair and subcutaneous soft tissue in the rat. The surface area to volume ratio of the rat foot is also much higher. Water at 48°C may then be more effective at raising the temperature of subdermal tissues in the rat, such that the noxious stimulus at such sites is equivocal to that achieved with water at higher temperatures in the cat. As 48°C was an adequate stimulus in the present study, higher temperatures were not employed to avoid causing actual tissue damage. Thus the conclusion that potentially severe or damaging noxious stimuli were needed to produce spinal release of irSP in the cat does not appear to be a cross-species generalisation, since such severe stimuli were not needed in the rat.

The small zones of irSP detection in the deep dorsal horn seen following nerve stimulation in the present study were not a consistent finding in the earlier cat studies. Although irSP containing terminals predominate in the superficial dorsal horn of both species, they are also seen in deeper laminae of the spinal cord. At these deeper sites the network of irSP terminals is less continuous, forming isolated populations of irSP containing terminals. Individual microprobe images from cat studies occasionally identified small zones of irSP release in the deep dorsal horn, but this was never consistent enough to reach significance in pooled data (personal observation). Deep irSP release in the rat was seen only with tibial nerve stimulation, not with noxious heat or pinch. This may reflect the spinal distribution of nerve terminals from joint afferents or 'silent' nociceptors in this species, i.e. those receptors not activated by noxious stimuli applied to normal skin.

In conclusion, the release of irSP following noxious afferent stimulation in the rat is limited to the superficial dorsal horn, and adequate stimuli to evoke such release are in the same range as those employed in cat studies, although lower grade thermal stimuli were adequate in evoking release in the rat. Additionally, the pattern of irSP release following such stimuli in the rat is comparable with that previously reported in the cat. By revealing this focal release with the antibody microprobe technique, we have demonstrated that microprobes are

a suitable experimental tool for use in smaller, as well as larger, laboratory species.

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MICROINJECTION OF NEUROPEPTIDE Y INTO THE SUPERFICIAL DORSAL HORN REDUCES STIMULUS-EVOKED RELEASE OF IMMUNOREACTIVE SUBSTANCE P IN THE ANAESTHETIZED CAT

A. W. DUGGAN,* P. J. HOPE and C. W. LANG

Department of Preclinical Veterinary Science, University of Edinburgh, Summerhall,
Edinburgh EH9 1QH, U.K.

Abstract—In barbiturate anaesthetized spinal cats, antibody microprobes were used to measure release of immunoreactive substance P in the superficial dorsal horn following electrical stimulation of unmyelinated primary afferents of the ipsilateral tibial nerve. Prior microinjection of neuropeptide Y (0.2–0.6 μ l of 10^{-5} mol/l solution) in the region of the substantia gelatinosa reduced the evoked release of immunoreactive substance P for up to 40 min. Microinjection of similar volumes of phosphate-buffered saline at similar sites was without effect.

This action of neuropeptide Y could contribute to analgesia, particularly if this neuropeptide is co-released with noradrenaline from axon terminals in the superficial dorsal horn.

The superficial dorsal horn of the spinal cord of several species contains a dense network of neuropeptide Y (NPY)-containing fibres, mainly of supraspinal origin.^{1,4,6,7,23,27} Many such fibres probably contain both NPY and noradrenaline since these substances extensively co-exist in brainstem neurons.^{15,24} The superficial dorsal horn of the spinal cord is a site of potential interaction of many neuroactive compounds. The substantia gelatinosa is the major site of termination of cutaneous unmyelinated primary afferents,³⁰ many of which are nociceptors, and these fibres contain excitant amino acids and neuropeptides either singly or in a complex series of combinations.¹⁴ In addition, both intrinsic neurons of the substantia gelatinosa as well the terminals of propriospinal and supraspinally derived fibres contain amino acids, monoamines and neuropeptides. To understand the interactions between such a wealth of compounds is clearly a complex task. One aspect which can be investigated experimentally is whether any of the substances contained within the substantia gelatinosa act to reduce the release of compounds from the central terminals of primary afferent fibres.

Both in the peripheral and central nervous systems there are several instances where NPY has been shown to have effects on nerve terminals consistent with an impairment of transmitter release.^{8,18,20,26,38} If NPY had such an action in the superficial dorsal horn, and if the relevant receptors were located near the terminals of nociceptive afferents, then such a mechanism could contribute to analgesia both of segmental and supraspinal origin. An inhibition

of transmitter release from the central terminals of nociceptors has been suggested as important in several mechanisms of analgesia. Thus, Melzack and Wall³² proposed a segmental presynaptic inhibition of nociceptive transmission which could be activated by impulses in large diameter (non-nociceptive) primary afferent fibres. Presynaptic inhibition, either directly by supraspinal fibres or indirectly through activation of segmental inhibition, has been proposed as a mechanism important in supraspinal control of spinal transmission of nociceptive information.^{2,10} NPY requires consideration in both contexts since analgesia has been demonstrated following its intrathecal administration to the rat.⁴¹

The antibody microprobe has shown that, in the anaesthetized cat, peripheral noxious cutaneous stimuli produce a release of immunoreactive substance P (IR SP) localized mainly to the region of the substantia gelatinosa.^{11,13} The present experiments have examined whether NPY modifies such release. Because the antibody microprobe can detect release from relatively small areas of the central nervous system,¹³ it was appropriate to administer NPY also to restricted areas. This was done by microinjecting NPY in approximately 0.2 μ l volumes from micropipettes positioned with their tips in the region of the superficial dorsal horn in which microprobes have localized evoked release of IR SP.

EXPERIMENTAL PROCEDURES

Antibody microprobes were prepared as previously described.¹² Briefly, fine glass micropipettes, heat sealed at both ends, were incubated in a 10% solution of aminopropyltriethoxysilane in toluene. This produced a siloxane polymer layer on the outer surface of microprobes and glutaraldehyde was then used to immobilize protein

*To whom correspondence should be addressed.

Abbreviations: IR SP, immunoreactive substance P; NPY, neuropeptide Y; PBS, phosphate-buffered saline.

A (Porton) to this polymer. Protein A then bound immunoglobulins present in a polyclonal antiserum containing antibodies directed at the carboxy terminus of SP (Peninsula Laboratories). These antibodies bind substance P and fragments of substance P containing the last five amino acids: there is negligible cross-reactivity with neurokinin A and neurokinin B (information supplied by the manufacturer).

Experiments were performed on seven cats anaesthetized initially by the intraperitoneal injection of pentobarbitone sodium (35 mg/kg) and anaesthesia was maintained by the continuous infusion of pentobarbitone, 3 mg/kg/h. All animals were artificially ventilated following neuromuscular paralysis with 4 mg/kg/h gallamine. Blood pressure was monitored via a cannula in a carotid artery and end tidal CO_2 levels were continually measured and maintained at 4%. The lumbar spinal cord was exposed by removal of the relevant laminae and the spinal cord was then transected at the thoracolumbar junction following injection of 0.1 ml of 2% lignocaine at the site of transection. The lumbar dura mater was cut longitudinally and retracted laterally. A thin layer of Ringer/agar was then placed over the dorsal surface of the exposed spinal cord. At sites of proposed microprobe insertion an area of agar was removed and a small part of the pia-arachnoid was removed with sterile fine forceps. The exposed dorsal area of the spinal cord was continuously irrigated with sterile Ringer solution held at 37°C by a heat exchanger jacket around the delivery tube. Cats received two intramuscular injections of ampicillin, 25 mg/kg, at approximately 10 h intervals.

Although IR SP release in the superficial dorsal horn is evoked by a variety of noxious peripheral stimuli,^{13,36} it is difficult to deliver such stimuli repeatedly without damaging peripheral tissues and hence the firing of nociceptors to successive stimuli may alter. For such a reason electrical stimulation of the tibial nerve was used to elicit spinal release of IR SP in the present experiments. Previous experiments have shown that low amplitude stimuli exciting mainly myelinated afferents of this nerve in the cat do not evoke a spinal release of IR SP but that stimuli also exciting unmyelinated afferents do produce release localized predominantly to the region of the substantia gelatinosa. The right and left tibial nerves were exposed in the calf, immersed in liquid paraffin and mounted on platinum stimulating electrodes. Stimulation employed square wave pulses, 0.3 ms duration, and an amplitude of at least 80 times the threshold stimulus needed to produce a short latency field potential, recorded with a microelectrode placed in the upper dorsal horn. Stimulus frequency varied from 10 to 30 Hz but was constant for each experiment and stimulus durations were all 20 min.

Microprobes were inserted into the spinal cord (two at a time) with stepping motor micromanipulators. With the first probe introduced into a particular area of the spinal cord, it was usual to obtain extracellular recordings during introduction to determine the areas of the skin of the hind limb which activated adjacent neurons during brushing. All microprobes were inserted to a depth of 3 mm which, in the lumbar dorsal horn of the adult cat, places the tips in the upper ventral horn. In a typical experiment four to eight microprobes were left in the left side of the spinal cord in the absence of peripheral nerve stimulation and then subsequent microprobes were associated with left tibial nerve stimulation. The stimulus was not delivered in the time required to remove one pair of microprobes and insert a fresh pair and this period lasted 5–10 min. After about 20 microprobes had been inserted into the left side of the spinal cord, the sequence was repeated on the right side.

The antibody microprobe technique detects bound endogenous ligand by the failure of binding of exogenous radiolabelled ligand. Thus, following removal from the spinal cord, microprobes were washed for 15 min in cold phosphate-buffered saline (PBS) containing Tween (0.1%) and then incubated for 24 h at 6°C in a PBS-azide solution

of ^{125}I -radiolabelled SP (Amersham) containing 0.5% bovine serum albumin. The final dilution resulted in approximately 2000 d.p.m. per μl . After this incubation, microprobes were washed for 15 min in PBS-Tween while continually drawing the solution through the patent tips to remove any radiolabelled SP from within. The tips were then broken off and glued to a sheet of paper, which was placed in an X-ray film cassette with a sheet of monoemulsion film (Kodak, NMC).

Images of microprobes were analysed with an image analysis system employing an Image Technology PC Vision frame grabber board operating in a Data Control Systems 286e (AT based) computer. A charge coupled device camera scanned each image and, as described previously,²² after background subtraction, a transverse integration of the optical density of the image of each microprobe was executed at defined intervals. With the magnification of the system used and the resolution of the image analysis system (512×512 locations per frame) this corresponds to a 10 μm interval for transverse integrations. The resultant integrals were stored on a hard disk record which included 40 coded values which described the experimental conditions relevant to that particular image. An analysis program subsequently obtained groups of microprobes which met stated criteria and obtained the mean image analysis of each group, which was plotted with respect to the distance within the spinal cord (see Fig 1–5). The spatial resolution of microprobe images is dependent on radiation scattering and hence on the distance between sources and film emulsion. At 3 mm from the tip, a microprobe has a diameter of approximately 30 μm and hence it is improbable that a biological resolution of 10 μm can be attained. Hence in subsequent mathematical treatments, the resolution was reduced to approximately 33 sites per mm by calculating means for three successive values. Each site is treated independently and hence, for a selected group of microprobes, a mean optical density (grey scale) can be calculated for each site and, when comparing two groups of microprobes, statistical significance can be assigned to differences between mean optical densities at each site.

Microinjection of NPY was performed in two ways. In two experiments the tip of an antibody microprobe and a micropipette containing 0.5 μl of NPY were positioned just above the dorsal surface of the spinal cord. The microprobe was held vertically in a micromanipulator; the NPY-containing micropipette was at an angle of 10°C to the vertical in an anteroposterior plane. After the micromanipulator positional readings had been recorded, the NPY-containing pipette was retracted and the microprobe introduced 3 mm into the spinal cord. A simple trigonometric calculation then determined the distance the NPY-containing pipette needed to be moved rostrally so that when introduced into the spinal cord, with its tip positioned 1.1 mm from the dorsal surface (this approximates to the middle of the substantia gelatinosa), the tip would be 100 μm from the shaft of the previously introduced antibody microprobe. NPY (0.2 μl) was then microinjected slowly approximately over the whole period the microprobe was in the spinal cord.

In the remaining five experiments a series of two to three microinjections, each of 0.2 μl and 1.0–1.2 mm from the spinal cord surface, were made with a micropipette successively introduced at the margins of an area denuded of pia mater. Microprobes were then inserted in the central part of such an area after the microinjections were complete.

The NPY-containing micropipettes had tip sizes of 15–20 μm and were calibrated by filling the shanks with volumes of firstly 0.2 and then 0.4 μl of water. These volumes were expelled from a Hamilton microlitre syringe onto a small piece of Parafilm and rapidly sucked up into a micropipette under microscopic control. Calibration marks were then placed on the micropipette with a fine pen and the water subsequently expelled and replaced by

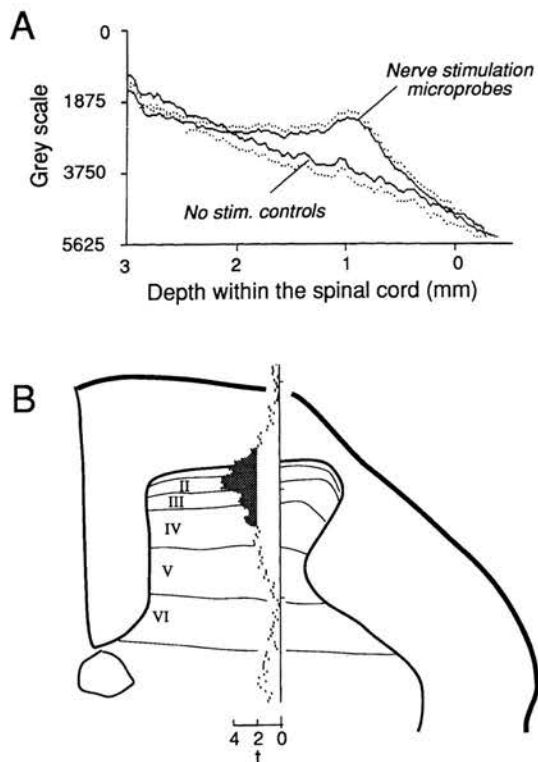


Fig. 1. Stimulus-evoked release of immunoreactive substance P. (A) The mean image analysis of 16 microprobes present in the spinal cord of the spinal cat for 20 min, without any peripheral nerve stimulation, is compared with that of 33 microprobes also in the spinal cord for 20 min, but throughout that time the ipsilateral tibial nerve was electrically stimulated at a strength adequate to excite both myelinated and unmyelinated fibres. Ordinate: integrals obtained during analysis of microprobe images. Optical density was measured in $30\ \mu\text{m}$ squares on a grey scale of 0–255 and transverse integrations then performed over each microprobe image. Abscissa: depth within the spinal cord with the surface being at 0. The lines join the mean grey scale values for the two groups of microprobes. For microprobes present in the spinal cord during nerve stimulation the (+) standard error of the mean grey scale value at each analysis point is plotted and for the no-stimulus microprobes the (–) standard error is plotted at each point. (B) A representation of the sites in the spinal cord where nerve stimulation produced significant increases in levels of immunoreactive substance P. The diagram was obtained from a transverse section of the seventh lumbar spinal segment. Roman numerals indicate the approximate positions of spinal laminae. At each analysis point in the mean image analyses shown in A the t statistic derived from the standard error of the difference of means has been calculated and plotted in relationship to position in the spinal cord (see text). The hatched area indicates the sites where the difference produced by nerve stimulation is significant at the $P < 0.05$ level.

an NPY-containing solution. In the spinal cord, NPY was microinjected by pressure applied to the interior of the micropipette by a hand-held syringe and tubing. The volumes injected were approximate in the sense that a graticule in a binocular microscope served to monitor the distance travelled by the upper level of the NPY-containing solution. It was possible to restrict the movement of this meniscus to one-tenth of the distance between the 0.2 and $0.4\ \mu\text{l}$ calibration marks, although the taper in the micro-

pipette shank complicates simple estimates of the volume ejected. The concentration of NPY in the microinjected solution was $10^{-7}\ \text{M}$ in one experiment and $10^{-5}\ \text{M}$ in six experiments.

A series of experiments was also performed in which PBS-azide was microinjected into the spinal cord using depths, volumes and injection times similar to those employed with microinjection of NPY. The injections of PBS were performed in the same animals used for microinjection of NPY. Such control injections were needed for two reasons. Firstly, a decline in the effectiveness of nerve stimulation in releasing IR SP could simply result from a relative exhaustion of levels of SP in nerve terminals. Secondly, although the volumes of NPY microinjected were small, microprobes measure release of a neuropeptide from very small areas of the central nervous system and hence any microinjection must be viewed as a distortion potentially altering release either by disrupting fibres and/or neurons, or causing some neurons to fire by mechanical deformation.

RESULTS

Stimulus-evoked release of immunoreactive substance P

The mean image analyses of 16 microprobes inserted 3 mm into the spinal cord and left for 20 min in the absence of peripheral nerve stimulation and 33 microprobes inserted for the same times and depths but during electrical stimulation of myelinated and unmyelinated primary afferents of the ipsilateral tibial nerve are compared in Fig. 1A. These microprobes are derived from all seven experiments. Significant differences between these mean image analyses occur from 0.6 to 1.4 mm from the dorsal surface of the spinal cord with the maximal increase occurring at 1.0 mm (Fig. 1B). This site of maximal release approximates to the region of the substantia gelatinosa and lamina I and agree with previously reported findings.¹³ It needs to be pointed out that when dye is ejected from the tip of a single microprobe, and the resultant deposit is subsequently located in a spinal cord section, then the sites of presence of a neuropeptide can be inferred with some accuracy. With mean image analyses as presented here, however, the location of sites of release is less precise. Thus with microprobes placed a fixed distance in the spinal cord (3 mm in the present experiments), the relationship of spinal laminae to distance from the tip will vary between animals and, in a given experiment, with distance from the midline. When encoding the information describing the position of each microprobe, a correction factor is included when appropriate, but nevertheless the referring of spinal laminae to a mean image analysis should be regarded as approximate.

Microprobes present in the spinal cord during microinjection of neuropeptide Y

The mean image analyses of 12 microprobes inserted 3 mm into the spinal cord for 20 min and with electrical stimulation of myelinated and unmyelinated afferents of the ipsilateral tibial nerve (stimulus controls) and 13 microprobes inserted under the same conditions but with NPY ($10^{-7}\ \text{M}$ in one experiment,

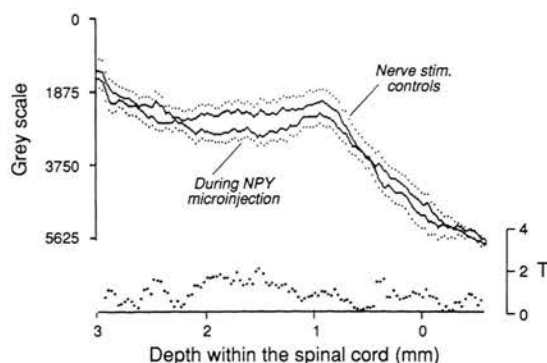


Fig. 2. Reduced evoked release of IR SP during microinjection of NPY in the superficial dorsal horn. The mean image analyses of two groups of microprobes are shown. All were inserted 3 mm into the spinal cord and remained there for 20 min while the tibial nerve was electrically stimulated. The upper trace is the mean image analysis of 12 microprobes inserted prior to microinjection of NPY; the lower trace is the mean image analysis of 13 microprobes present while NPY ($0.2 \mu\text{l}$) was slowly microinjected over the 20 min that microprobes were in the superficial dorsal horn. Both groups of microprobes are derived from the same experiments. A plot of the t statistics derived from the differences of the means of the two image analyses is shown with respect to distance from the surface of the spinal cord.

10^{-5} M in the other) being microinjected 1.1 mm from the cord dorsum and $100 \mu\text{m}$ from the microprobe shaft are compared in Fig. 2. Although the mean image analysis of microprobes inserted during NPY microinjection is displaced below that of control microprobes (indicating reduced stimulus-evoked release of IR SP), this only attains significance at about 1.5 mm from the dorsal surface. In the first 20 min period after microinjection, the inhibition of IR SP release was greater and results from these

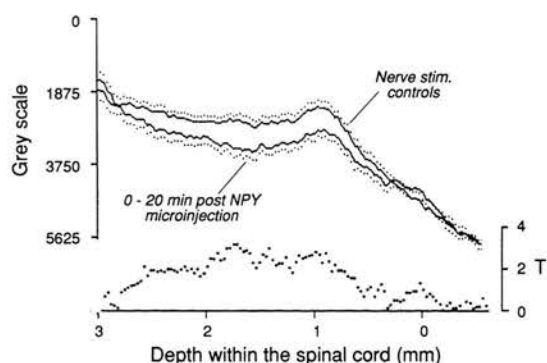


Fig. 3. Reduced evoked release of IR SP in the period 0–20 min after microinjection of NPY into the superficial dorsal horn. This figure compares the mean image analysis of 33 microprobes inserted during peripheral nerve stimulation, but prior to microinjection of NPY (these are derived from all experiments), and that of 22 microprobes present in the spinal cord in the period 0–20 min after microinjection of NPY in the superficial dorsal horn, and also with peripheral nerve stimulation (these probes are also derived from all experiments). All were inserted 3 mm into the spinal cord. The t statistics derived from the differences of the means of the two illustrated mean image analyses are plotted below.

microprobes and those inserted into the spinal cord in the second 20 min period after microinjection are analysed below.

Microprobes inserted into the spinal cord after microinjection of neuropeptide Y

The effectiveness of peripheral nerve stimulation in evoking release of IR SP prior to and in the 20 min period following microinjection of NPY (10^{-5} M) is compared in Fig. 3. The mean image analysis for microprobes inserted following NPY is significantly displaced below that of stimulus control microprobes beginning at 0.8 mm and extending to 2.0 mm from the dorsal surface. This is virtually over the whole of the dorsal horn. In the second 20 min period after microinjection of NPY the evoked release of IR SP was still reduced but over a more restricted area of the spinal cord. Figure 4 compares the mean image analyses of the microprobes inserted in this period with those inserted prior to NPY microinjection. The tibial nerve was stimulated with both groups. The post-NPY (second 20 min period) analysis is displaced below that of preinjection microprobes indicating inhibition of release but the plot of significances of differences between these analyses indicates that the significant differences were restricted to a zone approximating to laminae I and II. Five microprobes were inserted in the third ensuing 20 min postinjection period and no impairment of nerve-evoked release of IR SP was observed.

Release of immunoreactive substance P following microinjection of phosphate-buffered saline containing sodium azide

No inhibition of stimulus-evoked release of IR SP occurred following microinjection of PBS–azide in the region of the substantia gelatinosa. Figure 5A illustrates the mean image analyses of microprobes inserted during peripheral nerve stimulation but prior to microinjection of PBS–azide and microprobes inserted into the spinal cord in the first 20 min after microinjection. There is a small zone at the surface of the spinal cord with the latter group indicating enhanced release of IR SP, but within the spinal cord the two analyses are virtually identical. Figure 5B compares preinjection microprobes with those inserted in the second 20 min period after PBS–azide microinjection and again the image analyses of the two groups are virtually identical.

DISCUSSION

The present experiments have shown that microinjection of 0.2 – $0.6 \mu\text{l}$ of NPY (10^{-5} M), in the region of the substantia gelatinosa of the dorsal horn, reduces nerve stimulus-evoked release of IR SP. Interpretation of these results requires consideration of the constraints imposed by microinjection. It was unknown in the present experiments whether NPY would be inactivated quickly or slowly within the

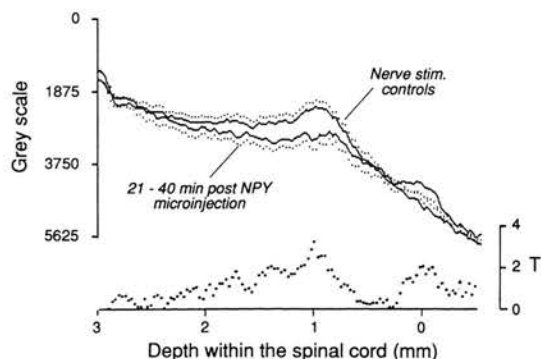


Fig. 4. Reduced evoked release of IR SP in the period 21–40 min after microinjection of NPY into the superficial dorsal horn. The nerve stimulus control analysis is for the same group of 33 microprobes shown in Fig. 3. The lower mean image analysis is for 20 microprobes present in the spinal cord with similar conditions to those of the control group but in the period 21–40 min after microinjection of NPY into the superficial dorsal horn. The t statistics derived from the differences of the means plotted in the two analyses are plotted below.

spinal cord and hence the initial experiments microinjected the peptide continuously while microprobes were in the spinal cord. This technique had the

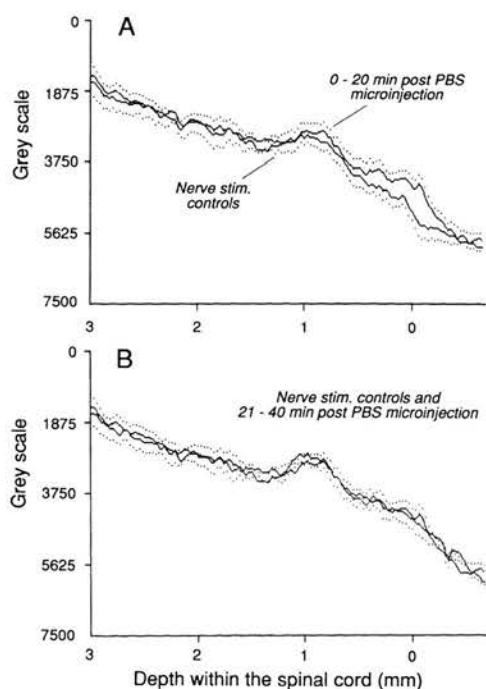


Fig. 5. Failure of microinjection of PBS in the superficial dorsal horn to reduce evoked release of IR SP. In both A and B the nerve stimulus controls comprise 25 microprobes present for 20 min in the spinal cord during electrical stimulation of the ipsilateral tibial nerve. In A, the mean image analysis of this group is compared with that of 12 microprobes inserted under similar conditions in the same experiments but 0–20 min after microinjection of PBS in the superficial dorsal horn; in B the comparison is with the mean image analysis of seven microprobes inserted 21–40 min after PBS microinjection.

disadvantage that the neuropeptide was administered at one site only (although relatively close to part of the microprobe shaft) and the time for diffusion was restricted when compared with the method of introducing microprobes after microinjection. The findings that greater inhibition of the evoked release of IR SP was produced by the latter method indicate its suitability for a compound such as NPY. Microinjection probably introduces relatively large amounts of a compound when compared with synaptic release, and hence local inactivation processes are likely to be saturated for some time after microinjection. Hence, even with a rapidly degraded compound, introduction of microprobes after microinjection is probably the preferred method. Thus persistence of the effect of NPY up to 1 h after microinjection does not necessarily represent the persistence of effect of this compound after synaptic release.

It is probable that most of the increases in IR SP produced by peripheral nerve stimulation in the present experiments came from release from the central terminals of peripheral nociceptors.^{11,13} Significant inhibition of IR SP release following NPY microinjection occurred over nearly the whole of the dorsal gray matter in the first 20 min after microinjection but was restricted to the region of laminae I–II in the ensuing 20 min. This suggests that control (no stimulus) microprobes were detecting a basal presence of IR SP in the deeper layers of the dorsal horn, that this was increased by nerve stimulation and that microinjection of NPY reduced both basal and stimulus-evoked release.

An inhibition of SP release from the central terminals of unmyelinated primary afferents can explain the present results. There are no fine structural studies of the relationship between NPY-containing fibres and the neural elements of the superficial dorsal horn of mammals, but one study in the lamprey⁵ found close apposition of IR NPY-containing varicosities and the ascending and descending branches of primary sensory neurons.

In the periphery several actions of NPY on nerve terminals have been described. There is evidence that NPY released from the sympathetic nerve terminals to blood vessels acts as a negative feedback to inhibit both its own release and that of noradrenaline.^{37,38} High intensity stimulation of the cardiac sympathetic nerves results in prolonged inhibition of the ability of the vagus to slow the heart. This effect, which is mimicked by NPY, is believed to result from an inhibition of acetylcholine release from vagal endings.²⁶ Guiliani *et al.*¹⁸ obtained evidence for an inhibition, by NPY, of tachykinin release from the peripheral terminals of sensory nerves to the airways of the guinea-pig.

There is also electrophysiological data supporting presynaptic actions of NPY in the central nervous system. In the hippocampus of the rat both Colmers *et al.*^{8,9} and Haas *et al.*²⁰ obtained evidence for an

inhibition of release of an excitatory transmitter, believed to be L-glutamate, by NPY (10^{-6} M), added to the perfusate of a slice preparation. It is important to emphasize that these latter effects were obtained in slice preparations (which lack a blood supply), since vascular effects need to be considered when any compound is injected into or superfused onto the brain or spinal cord under *in vivo* conditions. NPY does contract blood vessels³⁹ and hence a contribution of such an effect to the present results cannot be excluded. It is unknown whether small intrinsic vessels of the spinal cord bear receptors to NPY.

The dense plexus of NPY-containing fibres in the superficial dorsal horn of several species^{1,4,6,7,23,27} probably mainly represents an extensive co-existence of NPY with catecholamines. NPY co-exists with noradrenaline in many neurons of the locus coeruleus^{15,24} and hence it is probable that it is co-released with noradrenaline in the superficial dorsal horn. There is a dense network of noradrenaline-containing fibres^{3,19,31,40} in the dorsal horn. In addition, a small number of intrinsic spinal neurons contain NPY.^{27,35}

The effects of NPY in the present experiments could act synergistically to noradrenaline in controlling spinal transmission of nociceptive information. Thus, intrathecal administration of noradrenaline inhibits spinal reflexes to peripheral noxious stimuli^{25,28,34} and microiontophoretic administration of noradrenaline in the substantia gelatinosa suppresses the transmission of nociceptive information to deeper neurons.^{16,21,44} Noradrenaline has been shown to hyperpolarize neurons of the rat substantia gelatinosa⁴³ and hence a co-release of noradrenaline and NPY from the spinal terminations of supraspinally derived fibres could inhibit spinal transmission of nociceptive information by both pre- and postsynaptic mechanisms. Noradrenaline probably also acts presynaptically since studies in the spinal cord of the rabbit using push-pull cannulae have shown a reduction of noxious stimulus-evoked release of IR SP following superfusion of the spinal cord with noradrenaline.²⁹

Much of the work on noradrenaline and spinal transmission of nociceptive information was done in the absence of knowledge of the co-existence of noradrenaline and NPY. There is some evidence that spinal inhibition from activity in coeruleospinal fibres cannot be fully accounted for by release of noradrenaline. In both the rat and cat, inhibition of spinal neurons by noradrenaline is blocked by α -2 adrenoceptor antagonists such as idazoxan and not by α or β adrenoceptor antagonists.^{16,44} In the cat, however, iontophoretic administration of idazoxan in amounts adequate to block inhibition by noradrenaline failed to block spinal inhibition from electrical stimulation in the region of the locus coeruleus or nucleus Kolliker-Fuse.⁴⁴ Intravenous idazoxan also did not block this inhibition. One explanation for such a result is that the action of NPY was sufficient to effect inhibition despite blockade of the action of co-released noradrenaline.

Fuxe *et al.*¹⁷ have recently suggested differing functional roles for high and low affinity receptors for NPY. Low affinity receptors are proposed as being localized to subsynaptic areas where high concentrations of non-degraded peptide are encountered. High affinity NPY (possibly Y2 receptors) are proposed as occurring remotely from sites of release and dependent on diffusion of released NPY. They term the process "volume transmission". A proportion of released molecules could be degraded by peptidases and hence Y2 receptors are the likely candidates for mediating the remote effects of released NPY because of their activation by carboxy-terminus fragments 13–36 of NPY. High affinity sites for NPY have been demonstrated in the trigeminal nucleus substantia gelatinosa³³ and, hence, if the hypothesis of Fuxe *et al.*¹⁷ is correct, a somatopically diffuse inhibition of substance P release may follow synaptic release of NPY.

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Note added in proof:

A recent study [Bleakman D., Colmers W. F., Fournier A. and Miller R. J. (1991) *Br. J. Pharmac.* **103**, 1781–1789] found that NPY (10^{-7} M) inhibited calcium entry into cultured rat dorsal root ganglion cells and a Y_2 receptor was involved. This was proposed as underlying a previous report [Walker M. W., Ewald D. A., Perney T. M. and Miller R. J. (1988) *J. Neurosci.* **8**, 2438–2446] that NPY reduced high potassium evoked release of substance P from cultured rat dorsal root ganglion cells. Both of these studies are in accord with the present findings in the cat.



RELEASE OF IMMUNOREACTIVE GALANIN IN THE SPINAL CORD OF RATS WITH ANKLE INFLAMMATION: STUDIES WITH ANTIBODY MICROPROBES

P. J. HOPE,* C. W. LANG,† B. D. GRUBB‡ and A. W. DUGGAN§

Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, Scotland, U.K.

Abstract—Antibody microprobes bearing antibodies to the carboxy-terminus of rat galanin were inserted into the spinal cords of anaesthetized normal rats and those in which ankle inflammation had been induced by the unilateral subcutaneous injection of Freund's adjuvant four to six days previously. In normal rats, a basal presence of immunoreactive galanin was detected in the dorsal horn. Similar levels of immunoreactive galanin were found in the dorsal horn of both sides of the spinal cord in animals with unilateral ankle inflammation. Flexing the ankle or compressing the foot in normal rats failed to alter levels of immunoreactive galanin detected by microprobes. In animals with ankle inflammation, prolonged periods of ankle flexion did release immunoreactive galanin in the ipsilateral dorsal horn. Subsequent noxious ankle compression in these animals did not increase but rather decreased immunoreactive galanin in the dorsal horn to below basal levels.

The reason for this decrease is unknown but it may represent an inhibition of release or a depletion of spinal stores of galanin.

In the rat spinal cord, galanin occurs in dorsal root ganglion neurons, intrinsic neurons of the spinal cord and in fibres of supraspinal origin.^{1,9,12,15,20} In most studies of the possible functions of galanin in the spinal cord, this neuropeptide has been applied topically to the spinal cord surface.

When galanin has been administered in this way the effects have not been uniform. Following intrathecal administration in relatively low doses, galanin has been variously found to produce hyperalgesia to mechanical but not thermal noxious stimuli,² to result in vocalization to innocuous mechanical peripheral stimuli² and to enhance reflexes to peripheral noxious thermal but not mechanical stimuli.²³ In contrast to these enhanced responses, a number of depressant actions of intrathecal galanin have also been observed, although following the administration of larger amounts than those producing increased responses. These depressant actions include decreased responses in hot plate and tail flick tests¹⁷ and decreased flexor reflexes produced by peripheral

nerve stimulation.²⁴ In addition, galanin has been found to reduce the potentiation of flexor reflexes produced by conditioning stimulation of a peripheral nerve²⁴ and to markedly potentiate the depression of these reflexes by intrathecal morphine.²⁵

Following nerve crush in the rat, galanin levels in dorsal root ganglion cells (along with those of vasoactive intestinal polypeptide and neuropeptide Y) increase markedly^{8,21,22} and this is associated with altered effects of topical galanin. Thus, in normal rats, galanin reduced the facilitation of hindlimb flexor reflexes produced by substance P applied topically to the spinal cord, but this effect disappeared within 11–14 days of peripheral nerve section. Moreover, at this stage, galanin reduced facilitation of the flexor reflex by vasoactive intestinal polypeptide. Such experiments led Xu *et al.*²⁶ to propose that galanin released from primary afferents has a function during nerve regeneration to suppress spinal transmission of ectopic impulses arising from regenerating nerve endings.

Compounds released from the central endings of primary afferent fibres are normally thought of as important in excitatory transmission. These depressant actions of galanin do not appear compatible with such a role, although galanin could be co-released with an excitant amino acid and/or tachykinin, and have independent inhibitory actions. Alternatively, these depressant effects could represent the action of galanin released from intrinsic neurons of the spinal cord or from extrinsic fibres such as those descending from the brain stem.

*Present address: Department of Pharmacology, Syntex Research Centre, Heriot-Watt University Research Park, Riccarton, Edinburgh EH14 4AP, Scotland, U.K.

†Present address: Department of Physiology, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

‡Present address: Department of Cell Physiology and Pharmacology, University of Leicester, PO Box 138, Leicester LE7 9HN, U.K.

§To whom correspondence should be addressed.

Abbreviations: IR, immunoreactive; PBS, phosphate-buffered saline.

Experiments in this laboratory have had a continued interest in changes in the central release of neuropeptides with developing inflammation in peripheral tissues.^{10,19} We have extended this series to include galanin. Little is known of the role of galanin in inflammation, but Kuraishi *et al.*¹³ found that intrathecal administration of an antiserum to galanin induced hypoalgesia to mechanical stimulation of an inflamed but not a normal hind paw of the rat.

EXPERIMENTAL PROCEDURES

Experiments were performed on anaesthetized rats (female and male, 220–320 g). Both normal (untreated) rats ($n = 11$) and rats with a unilateral inflammation ($n = 16$) of the ankle were used.

Induction of peripheral inflammation

Under diethyl ether anaesthesia, rats (270–330 g) received four subdermal injections of Freund's complete adjuvant (Sigma, 1.0 mg/ml, total volume of 0.15 ml) around the ankle joint to induce a unilateral inflammation. This injection results in a unilateral swelling of the ankle region, and previous studies^{6,18} have shown that this involves both the joint and periarticular tissues, e.g. connective tissue, tendons and overlying skin. For the purposes of this paper, we shall describe this lesion as unilateral inflammation of the ankle. Four to six days after the induction of inflammatory lesions, the animals were used for neuropeptide release studies.

Microprobe preparation

Antibody microprobes were prepared as described previously.^{3,4} Fine glass micropipettes were heat sealed at both ends and incubated in a 10% solution of aminopropyltriethoxysilane in toluene. This produced a siloxane polymer on the outer surface of the microprobes, to which protein A (Sigma) was immobilized using glutaraldehyde coupling. Protein A then bound immunoglobulins present in an antiserum to rat galanin purchased from Peninsula Laboratories. Data from the manufacturer indicate negligible cross-reactivity with porcine galanin, neuropeptide Y and substance P. With microprobes bearing anti-galanin antibodies, however, preincubation in porcine galanin (10^{-7} M) for 30 min at 37°C resulted in greater than 60% suppression of [¹²⁵I]galanin. Preincubation with rat galanin (10^{-7} M) under the same conditions resulted in approximately 90% suppression of [¹²⁵I]galanin binding. Because antibody microprobes have to work within the constraint of antibody specificity, what is detected under *in vivo* conditions will be termed immunoreactive (IR) galanin. In contrast, no significant suppression of binding of [¹²⁵I]galanin was caused by preincubation of microprobes with 10^{-7} M endothelin, neuropeptide Y, substance P or neurokinin A. All incubations were carried out in 5 μ l capillaries previously treated with Sigmacote to prevent binding of peptides to glass.

Surgical preparation and experimental protocols

Anaesthesia was induced with intraperitoneal (i.p.) sodium thiopentone (120 mg/kg initially, maintained as required with 15 mg/kg i.p.). Blood pressure was monitored via a cannula in a carotid artery. Cannulae were also inserted into the trachea and jugular vein. Animals were mounted in a frame by means of three pairs of lateral bars applied to the vertebral bodies adjacent to the exposed segments of the spinal cord. A controlled heating blanket maintained body temperature at 36–38°C. Humidified oxygen was delivered to the region of the tracheal cannulae external opening with all animals. The lower laminae of the thoracic/upper lumbar vertebrae were removed and the

exposed spinal cord then covered with 3% agar in saline. To facilitate microprobe insertion, an opening was made in the agar forming a pool which was irrigated with warm sterile Ringer's solution. Within this pool, the dura mater was cut and retracted laterally. Unlike experiments on the cat,^{5,10,19} it was not necessary to remove the pia arachnoid in the rat to allow microprobes to be inserted into the spinal cord. In each experiment a proportion of microprobes was filled with Pontamine Sky Blue after breaking the tips to 5–10 μ m diameter. Pairs of microprobes aligned in the same rostrocaudal line and separated by 3 mm were inserted into the spinal cord with a stepping motor micromanipulator to a depth of 2.25 mm. They remained in the spinal cord for 10, 15 or 20 min. In the results as presented, microprobe images from all time periods have been pooled. More than 20 microprobes were inserted into the spinal cord in each experiment.

Peripheral stimuli used

Because the most likely stimulus producing pain from inflamed skin, joints or tendons is mechanical, resulting either from direct physical contact or movement of the damaged tissues, the present experiments employed a series of repeated mechanical stimuli of increasing intensity. These consisted of flexing the inflamed joint area and then mechanically compressing the inflamed tissues. Comparable stimuli were also applied to the uninflamed hind limbs or normal animals. Flexion of the ankle was applied by holding the hind paw gently and pulling the limb out to its full extent, then pushing the paw gently until it was in close apposition to the knee. This flexion of the ankle joint was repeated 10 times per minute throughout the period that microprobes remained in the spinal cord. The second (more severe) stimulus consisted of laterally compressing the ankle with a modified spring clip for 10 s each minute for the period that microprobes were present in the spinal cord.

Following removal from the spinal cord, microprobes were washed for 15 min in cold phosphate-buffered saline (PBS) containing 0.1% Tween and then incubated for 24 h at 4°C in a solution of [¹²⁵I]galanin (rat, 2000 c.p.m./ μ l). After this incubation, microprobes were washed for 15 min in cold PBS. The distal portions (8–12 mm) were then broken off and placed in an X-ray film cassette with a sheet of monoemulsion film (Kodak, NMB).

Details of the analysis of microprobe images have been published previously.^{3,7} Briefly, X-ray film images of microprobes were analysed with an image analysis system employing an Imaging Technology PC Vision Plus frame grabber board operating in a DCS 286e (AT-based) computer. A CCD camera scanned each image and, after background subtraction, a transverse integration of the grain density of the image of each microprobe was executed at defined intervals. With the magnification of the system used and the resolution of the image analysis system (512 \times 512 locations/frame), this corresponds to a 10 μ m interval for transverse integrations. The resultant integrals were stored on a hard disk with 37 coded values which described the experimental conditions relevant to that particular microprobe image. An analysis program subsequently obtained groups of microprobes which met defined criteria and determined the mean image analysis of each group, which was plotted with respect to depth within the spinal cord. In addition, differences between two defined groups of microprobes were assigned statistical significance by determining the *t*-statistics derived from the differences of means at each analysis point (see Figs 2 and 3). It is important to emphasize the spatial resolution of the method. Microprobe images were analysed at 100 sites/mm, but in the data of the present work this has been reduced to 33 sites/mm by obtaining the mean of three consecutive values. At the end of each experiment, Pontamine Sky Blue (contained in some microprobes) was ejected electrophoretically at defined depths

from the dorsal surface of the spinal cord. The cords were removed, processed and sectioned for determination of the location of the resultant dye spots.

RESULTS

Basal levels of immunoreactive galanin in normal rats and those with unilateral ankle inflammation

Comparisons between the mean image analyses of *in vitro* microprobes not exposed to galanin and those inserted into the spinal cord in the absence of any active peripheral stimulus (no stim.) showed a basal presence of IR-galanin in both sides of the spinal cords of normal rats and those with unilateral ankle inflammation. Further analysis showed that there were no significant differences in basal extracellular levels of IR-galanin in the dorsal horns and dorsal columns of normal ($n = 39$) and arthritic ($n = 58$) animals, and the ipsilateral ($n = 29$) and contralateral ($n = 29$) sides of the spinal cord of animals with peripheral ankle inflammation also showed no significant differences in basal levels of IR-galanin. Thus, comparable basal levels of IR-galanin were found in normal rats and animals with unilateral peripheral inflammation. Figure 1A compares the mean image analysis of microprobes inserted into the side of the spinal cord ipsilateral to peripheral inflammation, but in the absence of any added stimulation with that of *in vitro* microprobes. Figure 1B plots, in 30 μm intervals, the differences between the mean image analyses of the two groups and the hatched area indicates where these differences are significant at the $P < 0.05$ level. This shows that there was a basal presence of

IR-galanin at significant levels throughout the whole of the dorsal horn and dorsal columns, but not within the ventral horn.

Mechanical stimulation of normal and inflamed ankle joints and adjacent tissues

Animals with unilateral ankle inflammation. As described in Experimental Procedures, it was usual to insert two microprobes into the sides of the spinal cord ipsilateral to the peripheral ankle inflammation and flex the inflamed ankle for a defined period and, following their removal, to insert two microprobes into the opposite side of the spinal cord and flex the normal ankle in a similar manner. This sequence was repeated three or more times.

When the inflamed ankle was flexed, extracellular levels of IR-galanin were not increased above pre-stimulus levels during the first two periods of ankle flexion, but were increased by the third period. In Fig. 2A, it can be seen that the mean image analysis of microprobes inserted into the side of the spinal cord ipsilateral to the peripheral inflammation, but prior to ankle flexion, is virtually identical to that of microprobes present in the spinal cord during the first period of ankle flexion. Such a result also occurred during the second period of ankle flexion (not illustrated). Figure 2B and C, however, shows that during the third period of ankle flexion, extracellular levels of IR-galanin were significantly elevated in the superficial and deep dorsal horn when compared with those present in the absence of added stimulation.

Increasing the intensity of mechanical stimulation of inflamed tissues by laterally compressing the

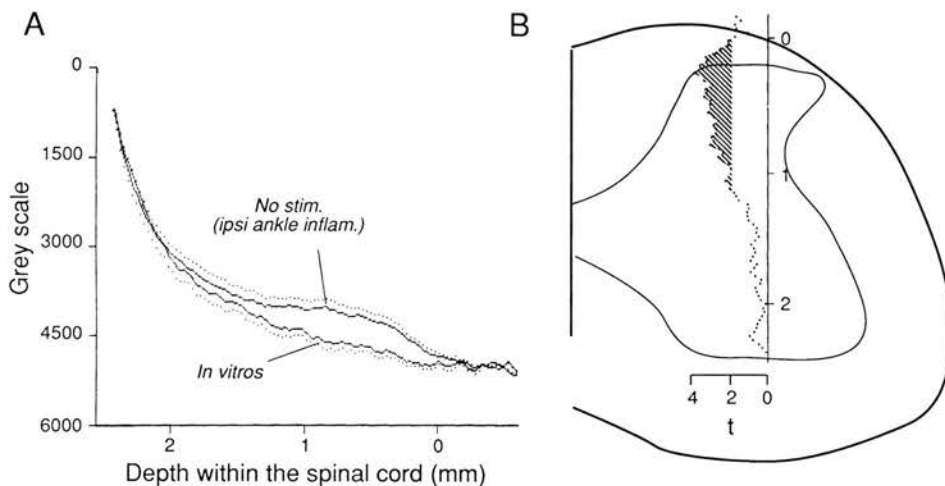


Fig. 1. Basal presence of IR-galanin in the side of the spinal cord ipsilateral to ankle inflammation. (A) The mean image analyses of two groups of microprobes are plotted with respect to length: those which were not inserted into the spinal cord but simply incubated in [^{125}I]galanin (*in vitro*, $n = 25$) and those remaining in the spinal cord in the absence of added peripheral stimulation (No stim., ipsilateral ankle inflammation, $n = 29$). With each mean image analysis, the mean grey scale was determined in 30 μm intervals and a line joins these points. At each point \pm S.E.M. is also plotted. (B) A plot of the t statistics derived from the differences between the two mean image analyses of A is related to an outline of the spinal cord at the area sampled. The hatched areas indicate where these differences are significant at the $P < 0.05$ level.

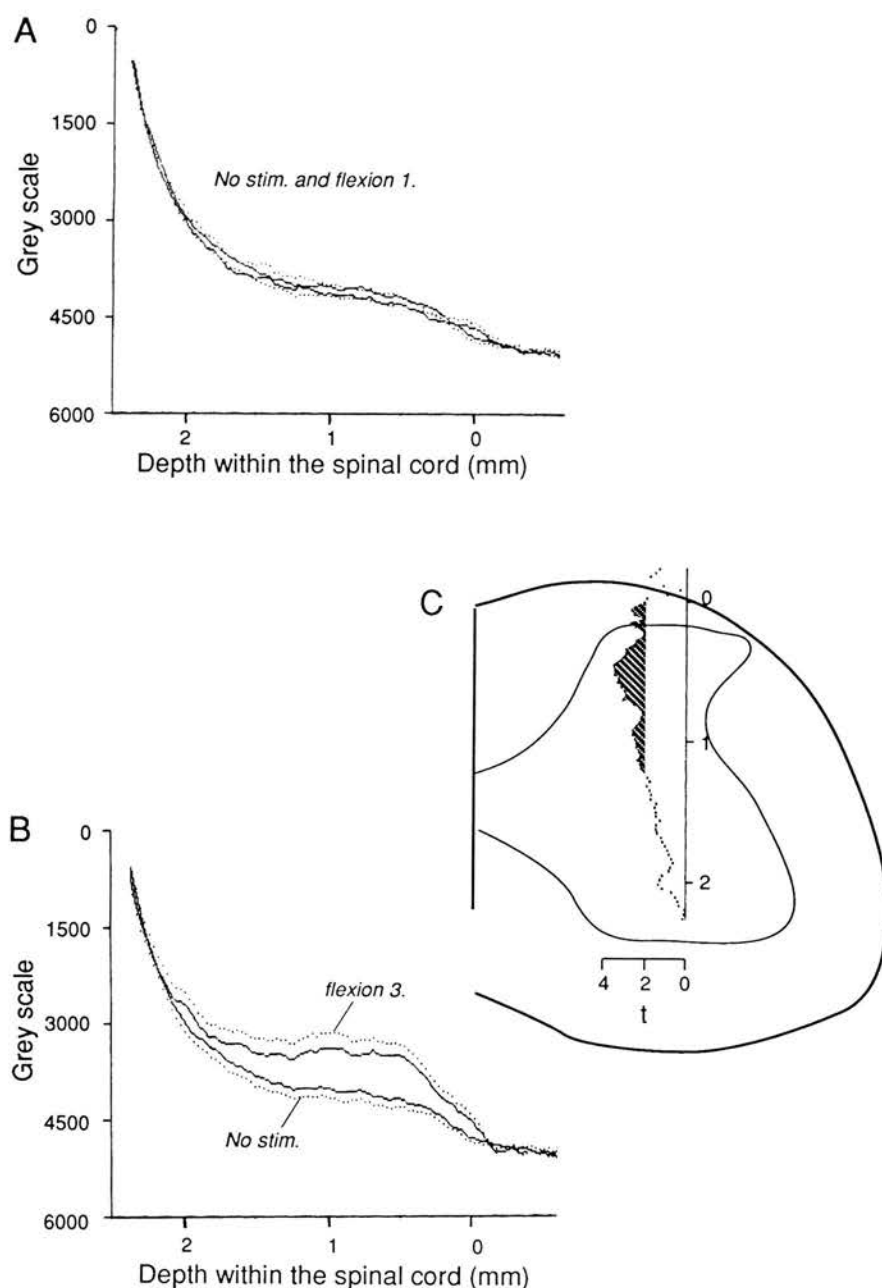


Fig. 2. Flexion of the inflamed ankle and spinal release of IR-galanin. (A) The mean image analysis of 29 microprobes present in the spinal cord in the absence of any added stimulus (No stim.) is compared with those in the spinal cord during the first period of flexion of the inflamed ankle ($n = 20$, flexion 1). (B) Microprobes from the third ($n = 13$, flexion 3) period of ankle flexion are compared with those present prior to ankle flexion (No stim., $n = 29$). (C) The differences between the No stim. and third flexion groups are plotted with respect to a diagram of the spinal cord. The hatched areas indicate where these differences are significant at the $P < 0.5$ level.

inflamed ankle gave the unexpected result of decreasing extracellular levels of IR-galanin. This decrease was not significant during the first period of noxious ankle compression, but was very evident during the second application of this stimulus. Thus, Fig. 3A compares the mean image analysis of microprobes present in the spinal cord during the second period of

ankle flexion and that of microprobes present in the absence of active peripheral stimulation. Figure 3B shows that significant differences occurred between these groups at several sites in the nucleus proprius of the dorsal horn.

Limited data were obtained on the effects of ankle compression on the normal side of animals with

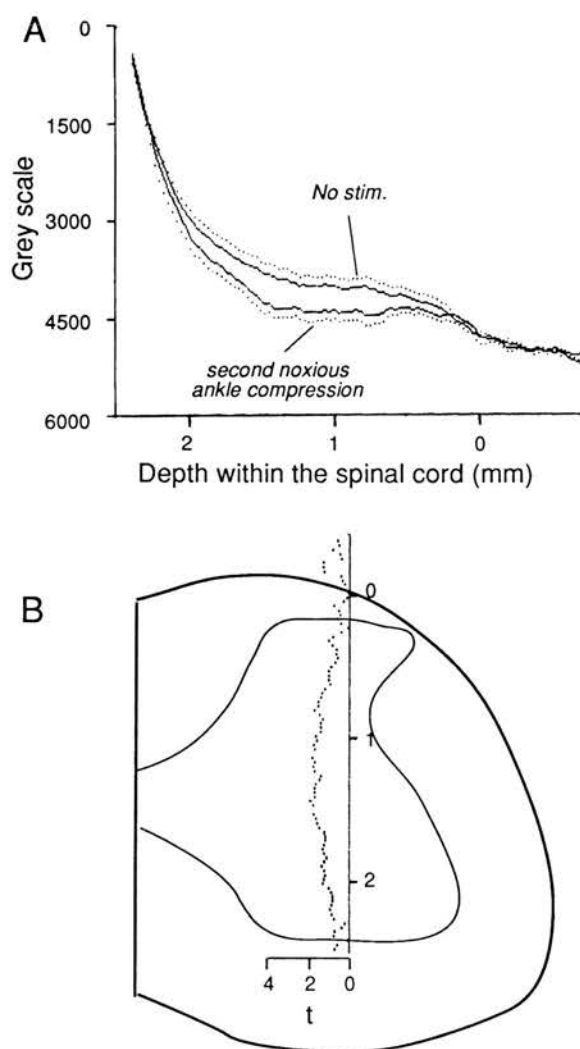


Fig. 3. Noxious lateral compression of the inflamed ankle region and spinal release of IR-galanin. (A) The mean image analysis of 29 microprobes present in the spinal cord in the absence of any added stimulus (No stim.) is compared with those present during the second ($n = 12$) period of noxious lateral ankle compression. (B) The differences between the two groups of microprobes in A are plotted with respect to depth in the spinal cord. Because only isolated points attain significance at the $P < 0.05$ level, there is no hatching as in Figs 1 and 2.

unilateral inflammation. Thus, the mean image analysis of seven microprobes present in the side of the spinal cord contralateral to the inflamed ankle and during compression of the normal ankle failed to show significant differences from that of microprobes present in the same side of the spinal cord and during flexion of the normal ankle (not illustrated).

Normal animals. Flexing the ankle of normal rats failed to increase extracellular levels of IR-galanin in the ipsilateral spinal cord (five microprobes). Severe compression of the foot of the normal rat also failed to alter basal levels of IR-galanin in the ipsilateral spinal cord (33 microprobes).

DISCUSSION

The present experiments found comparable basal levels of IR-galanin in both normal animals and in those with peripheral inflammation, but in both groups release of this neuropeptide was not easily produced by peripheral stimuli and, indeed, decreased extracellular levels followed noxious stimulation of inflamed tissues.

The source and stimulus for a basal extracellular presence of IR-galanin can only be conjectured, since this peptide is present in primary afferent fibres, intrinsic neurons and supraspinally derived fibres of the rat.^{1,9,11,12,15,20} There is dispute among investigators on the abundance of galanin-containing neurons in dorsal root ganglia of the rat with estimates varying from rare ($< 5\%$)²¹ to more common than neurons containing calcitonin gene-related peptide.¹¹ In the present experiments, release of galanin by peripheral stimuli was not readily detected. Thus, in rats with peripheral inflammation, repeated flexion of the involved ankle was needed to increase extracellular levels of IR-galanin and in normal rats noxious foot compression did not produce a spinal release of the neuropeptide. We do not believe this is the release pattern of a compound present in many primary afferent fibres. Release from intrinsic neurons of the cord and/or supraspinally derived fibres may be significant with galanin. The study of Morton and Hutchison,¹⁶ using antibody microprobes in the spinal cat, may be interpreted as showing a basal presence of galanin derived from intrinsic neurons of the spinal cord. In this work, a basal presence of IR-galanin was detected in the region of lamina II and at the spinal cord surface. Electrical stimulation of both A and C fibres of the tibial nerve (10 Hz) failed to produce a spinal release of IR-galanin. Similarly ineffective were peripheral noxious thermal and mechanical stimuli.

Using immunocytochemical techniques, galanin-containing neural structures in the spinal cord have been shown to be relatively heavily concentrated in laminae I and II of the dorsal horn, but not in the deeper laminae IV and V nor in the dorsal columns.²⁰ In the present experiments, this difference was not observed and IR-galanin was present relatively diffusely in the dorsal columns and dorsal horn, although peak levels were in the superficial dorsal horn. This may represent a diffusion of galanin away from sites of release, which implies a relative resistance to degradation at sites of release. Relevant to this is the report that, when incubated with membrane preparations of rat hypothalami, the half-lives of galanin 1–29 and galanin 1–16 were 100 and 28 min, respectively.¹⁴ If this reflects the rate of degradation of galanin under *in vivo* conditions, then diffusion away from sites of release is to be expected.

The results of noxious lateral ankle compression are unlike any we have observed with other neu-

ropeptides. It is possible that the decreased levels produced by this stimulus represent an inhibition of both basal and evoked release of galanin by the activity of afferents brought into play by such stimuli. An alternative explanation is that repeated stimuli deplete the stores of releasable galanin to the extent that basal levels decline below those present prior to any active stimulus. The report of Klein *et al.*¹¹ that 30 min of peripheral nerve stimulation in the rat resulted in reduced levels of IR-galanin, measured by immunocytochemistry of the dorsal horn, supports such a proposal. Both an inhibition of release or a depletion of stores as explanations for the present results require that previously released galanin be degraded approximately within the interval between insertion of successive microprobes.

CONCLUSIONS

The results of the present experiments are supportive of those of Kuraishi *et al.*¹³ in suggesting that

galanin has a functional role in the spinal cord, with the development of peripheral inflammation. Release studies alone cannot determine function and, in the present instance, whether released galanin facilitates or inhibits transfer of information from nociceptive primary afferents. The relative difficulty with which galanin was released by peripheral stimulation argues against this neuropeptide being a simple transmitter released by a particular population of afferent fibres. If galanin has an inhibitory role in spinal processing of nociceptive information, then its apparent depletion or inhibition of release by prolonged severe stimuli may have implications for the perception of pain with persistent inflammatory states.

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Effect of peptidase inhibition on the pattern of intraspinally released immunoreactive substance P detected with antibody microprobes

A.W. Duggan^a, H.-G. Schaible^b, P.J. Hope^a and C.W. Lang^a

^aDepartment of Preclinical Veterinary Sciences, University of Edinburgh Summerhall, Edinburgh (UK) and ^bDepartment of Physiology, University of Würzburg, Würzburg (Germany)

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Key words: Antibody microprobe; Immunoreactive substance P release; Peptidase inhibitor

Antibody microprobes bearing antibodies to the C-terminus of substance P (SP) were used to measure release of immunoreactive (ir) SP in the dorsal horn of barbiturate anaesthetized spinal cats. Electrical stimulation of unmyelinated primary afferents of the ipsilateral tibial nerve produced a relatively localised release of ir SP in the superficial dorsal horn. Prior microinjection of the peptidase inhibitors kelatorphan and enalaprilat in the dorsal horn resulted in ir SP being detected over the whole of the dorsal horn and the overlying dorsal column. This pattern had previously been observed with evoked release of ir neurokinin A and supports the proposal that a slow degradation results in a neuropeptide accessing many sites remote from sites of release.

INTRODUCTION

The encoding of the onset and offset of a burst of afferent information to a neurone requires that released neurotransmitters should be rapidly inactivated. If the inactivating mechanism is a component of the pre- and/or postsynaptic elements of a synapse, such an arrangement favours rapid inactivation and also minimises the probability that significant amounts of released transmitter will diffuse to adjacent areas. Thus active uptake into both the releasing terminal and adjacent glial processes, such as occurs with the inhibitory amino acid γ -aminobutyric acid, GABA^{10,20}, probably ensures that GABA has little direct effect on sites outside a synapse at which release has occurred. With neuropeptides the situation is less clear. High affinity uptake systems for neuropeptides have not been demonstrated in nervous tissue²⁵. Although enzymes are assumed to be important in limiting the actions of synaptically released neuropeptides, some reviewers have drawn attention to difficulties inherent in such an assumption and in particular to the failure of peptidases to be located adjacent to some sites of peptide release^{2,25}. Thus there is reason to believe that some neuroactive compounds released in the central nervous system will not be degraded rapidly and thus could access receptors remote from sites of release. The process whereby this widespread presence of discretely released compounds results in alterations in excitability of many

neurones has been termed volume transmission by Fuxe et al.¹¹.

Our interest in such a process comes from studies of the release of substance P (SP) and neurokinin A (NKA) in the spinal cord using antibody microprobes. These two compounds extensively coexist in primary afferent fibres³ and it was anticipated that their release would occur at similar sites in the spinal cord and in response to similar peripheral stimuli. Peripheral noxious cutaneous stimuli did evoke release of both neuropeptides, but whereas that of immunoreactive (ir) SP was centred on the substantia gelatinosa⁷ ir NKA was detected diffusely over the dorsal horn⁸. Because in vitro data indicate a relative resistance of NKA to enzymic degradation^{14,27,30} it was proposed that the ability of NKA to access sites remote from those of release resulted from a slow rate of degradation under physiological conditions. Such a finding has important implications for the possible functions of substance P and neurokinin A in the spinal cord and for the volume transmission proposal of Fuxe and Agnati¹¹.

One test of such a hypothesis is whether protecting SP from enzymic degradation results in the release pattern of this neuropeptide becoming similar to that of NKA with widespread diffusion in the spinal cord. Under in vitro conditions SP is degraded by a number of enzymes including aminopeptidases, endopeptidases and carboxypeptidases^{12,21,23,34}, but it is uncertain which of these are

important under physiological conditions and, in particular, in the central nervous system. The present experiments have used the antibody microprobe technique to examine the distribution of stimulus-evoked release of ir SP in the spinal cord of the cat following the administration of peptidase inhibitors.

It was decided that the combination of kelatorphan and enalaprilat should be used initially, since the former compound is an inhibitor of aminopeptidases, dipeptidyl-aminopeptidases and the neutral metallo-endopeptidase enkephalinase, EC 3.4.24.11³² and the latter inhibits peptidyl dipeptidase A (angiotensin-converting enzyme EC 3.4.15.1)^{28,31}. Since the antibody microprobe technique permits measurements of release at discrete sites in the central nervous system, it is appropriate that drugs attempting to modify release should also be administered at discrete sites of release. In the present experiments, therefore, kelatorphan and enalaprilat were microinjected from glass micropipettes with tips positioned in the substantia gelatinosa of the dorsal horn of the spinal cord and adjacent to sites of penetration by microprobes. The results indicate a remarkable diffusion of ir SP within the spinal cord following peptidase inhibition.

MATERIALS AND METHODS

Microprobe preparation

Antibody microprobes were prepared following the technique recently described⁶. Conventional glass micropipettes were heat sealed at both ends, incubated in a 10% solution of aminopropyltriethoxysilane in toluene and then heat cured. This procedure produced a siloxane polymer on the outer surface of each micropipette to which protein A was immobilised using glutaraldehyde. Protein A then bound antibodies to substance P during incubation of the micropipettes in a substance P antiserum. The antiserum used was polyclonal and directed against the C-terminus of SP. The manufacturer (Peninsula) indicated for this antibody significant cross-reactivity with SP 3–11, little for SP 4–11 and no cross-reactivity with NKA, neuropeptide K or neurokinin B. In vitro tests showed uniform binding of ¹²⁵I-SP (Bolton-Hunter) to the probes and an approximately 50% suppression of such binding after preincubation in a 10⁻⁷ M SP solution for 30 min at 37°C. The binding was not suppressed by preincubation in a Ringer solution with kelatorphan (10⁻³ M) or kelatorphan and enalaprilat (10⁻³ M each).

Animal preparation

The experiments were performed on anaesthetised, spinalised cats. Anaesthesia was induced with 35 mg/kg sodium pentobarbitone i.p. and, after cannulation of a cephalic vein, maintained by continuous infusion at 3 mg/kg/h. Blood pressure was measured with a cannula in a carotid artery. A tracheostomy was made for artificial respiration which was started after the dissection and following neuromuscular paralysis with gallamine administered intermittently at a dose of 4 mg/kg/h. End tidal CO₂ levels and body temperature were controlled throughout the experiment.

An extended laminectomy exposed the caudal lumbar spinal segments. The spinal cord was transected at the thoracolumbar junction following injection of 0.1 ml of 2% lignocaine. The cats were fixed in a metal frame. After cutting the dura, a layer of sterile Ringer-agar was placed over the dorsal surface of the spinal cord. At proposed insertion sites of the probes, the agar was removed and the pia-arachnoid was opened with fine forceps. A perfusion

pump delivered sterile Ringer solution continuously to the exposed areas. A heat exchanger jacket around the delivery tubing maintained this solution at 38°C. For electrical stimulation the tibial nerve of the left hindlimb was dissected after incision of the skin and separating the two heads of the gastrocnemius-soleus muscle. A small pool was formed with the skin flaps and filled with mineral oil and a stimulation electrode was placed under the nerve for en passant stimulation.

Experimental protocol

Before microprobes were inserted into the spinal cord, extracellular recordings were obtained in spinal laminae IV–VI to determine the distribution of afferent input from the tibial nerve and the hind paw to select sites for the study of ir SP release by microprobes. Pairs of microprobes were introduced to a depth of 4 mm using two stepping motor micromanipulators and kept in the cord for 10 min. During these periods either no stimulus was applied or electrical or noxious mechanical stimulation was performed. For nerve stimulation, electrical pulses of 0.5 ms duration and 50 V amplitude were applied with a frequency of 20 Hz to the ipsilateral tibial nerve. This stimulation was considered supramaximal for all afferent fibres in the tibial nerve. For noxious mechanical stimulation five crocodile clips were applied to the toes of the ipsilateral hind paw for periods of 3 min and this stimulus repeated once within the 10 min microprobes were in the spinal cord. First the left side of the cord and electrical nerve stimulation were used and then we switched to the right side of the cord and noxious mechanical stimulation was employed.

Prior to injecting peptidase inhibitors (control period) several probes were inserted in the absence of stimulation or during electrical nerve stimulation or noxious mechanical stimulation of the toes. Peptidase inhibitors were then microinjected into the grey matter using a micropipette connected to a syringe. Ringer solution containing peptidase inhibitors (up to 10⁻³ M) was injected at depths of 2.5, 2, 1.5 and 1 mm from the dorsal surface and around the region where the microprobes had been introduced. The volume of each injection was approximately 0.05–0.1 µl with total volumes of 0.2–0.5 µl. The solution contained either kelatorphan (a gift of Dr B.P. Roques) or a mixture of kelatorphan and enalaprilat. In some experiments only Ringer solution was microinjected. Following the microinjections, further probes were inserted at defined intervals (starting 15 min after the microinjections were complete) and the electrical or mechanical stimuli were repeated.

At the end of each experiment pontamine Sky blue (contained in microprobes) was ejected electrophoretically at depths of 1 and 4 mm from the dorsal surface of the spinal cord. The cords were removed, processed and sectioned for determination of the location of the resultant dye spots. These data were used to relate locations on the probes to the laminae of Rexed (see next paragraph).

Treatment of probes and data analysis

After removal from the spinal cord, microprobes were washed for 15 min in cold phosphate-buffered saline (PBS) containing Tween (0.1%) and then incubated for 24 h at 6°C in a PBS-azide solution of ¹²⁵I-Bolton-Hunter SP containing bovine serum albumin. The final dilution of the radiolabelled peptide was approximately 2000 dpm per µl. Following incubation in labelled peptide, the probes were washed for 15 min in PBS-Tween (0.1%), the tips were broken off, glued to a sheet of paper and placed in an X-ray cassette with a sheet of monoemulsion film for up to 7 days.

The resulting X-ray images were scanned in 10 µm intervals using a charge-coupled device camera and an image analysis system employing an Imaging Technology PC Vision Plus frame grabber board operating in a DCS 286e (AT based) computer¹³. This procedure produced an image of each probe showing the grey density (on a scale of 0 to 255) along 5 mm of the tip. Transverse integrations were then performed along each analysed image to give a plot of total optical density (grey scale) with respect to length. Although the image analysis system produced a plot with a longitudinal res-

olution of 10 μm , this was reduced to 30 μm by calculating the average of three successive integrals. This is more in line with the biological resolution of the microprobe method (see ref. 5). Inhibition of binding of radioactive SP on these probes (reduced grey density) indicates previous binding of ir SP in the cord. These areas of reduced binding were then related to sites within the cord (see above). Groups of probes were pooled and then means (\pm S.E.M. in 30 μm steps) were displayed in graphs which showed the averaged grey densities along the probes in relationship to the depth within the cord. Comparisons were made between different groups of probes using the Student's *t*-test at intervals of 30 μm .

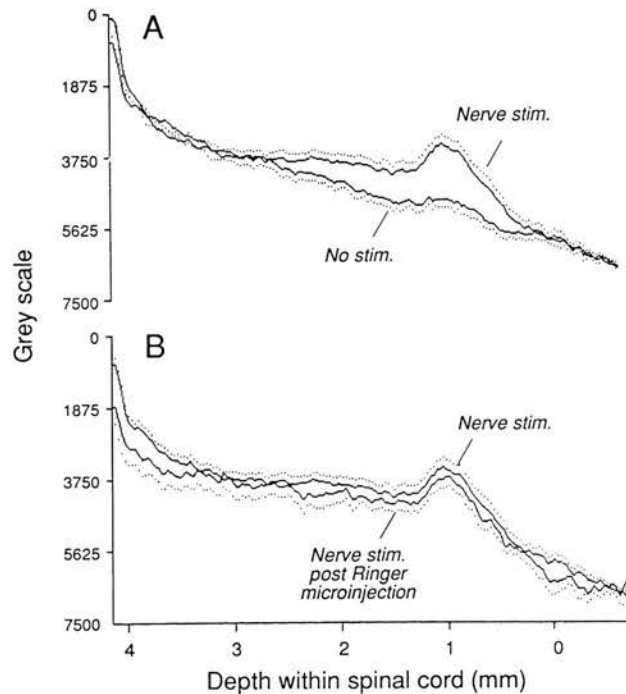


Fig. 1. Release of immunoreactive substance P in the dorsal horn following peripheral nerve stimulation and the failure of microinjection of Ringer solution to alter such release. A: images of 2 groups of microprobes present in the cord prior to nerve stimulation ('No stim.', $n = 19$) and during electrical stimulation of the tibial nerve ('Nerve stim.', $n = 42$). Each continuous line represents the mean densitometric analysis of a group of microprobes which have bound [^{125}I]substance P and produced autoradiographic images. The analysis was performed in 30 μm intervals and the dots represent the standard errors of the mean at each analysis point. Prior to incubation in [^{125}I]SP the probes were inserted 4 mm into the cord for periods of 10 min. Abcissa: depth in the spinal cord, 0 corresponds to the surface of the cord, 4 mm to the ventral border of the ventral horn and 1 mm approximates to lamina II. Ordinate: grey scale integrals obtained by transverse analysis of microprobe images in 30 μm steps. The slope of the 'No stim.' probes reflects the shape of the microprobes with an increasing number of binding sites along the shaft progressing away from the tips. Inhibition of binding of [^{125}I]SP (reduced grey density) is equated with previous binding of (unlabelled) endogenous ir SP in the cord. B: the mean image analyses of two groups: the 'Nerve stim.' group shown in A and microprobes present in the spinal cord during electrical stimulation of the tibial nerve, but post-microinjection of Ringer solution at depths 1.0, 1.5, 2.0 and 2.5 mm from the dorsal surface ('Nerve stim. post-Ringer microinjection', $n = 14$).

RESULTS

As previously reported⁷, electrical stimulation of the tibial nerve evoked a focal release of ir SP in the superficial dorsal horn of all cats. This is demonstrated in Fig. 1A. The 'No stim.' group displays the averaged image of 19 microprobes which were in the cord for periods of 10 min prior to electrical nerve stimulation. There is regular binding of [^{125}I]SP along the probe and the slope of the grey density curve was similar to that of probes incubated in labelled SP without previous exposure to the non-labelled neuropeptide. By contrast the averaged image of 42 probes present in the cord during electrical nerve stimulation (the 'Nerve stim.' group in Fig. 1A)

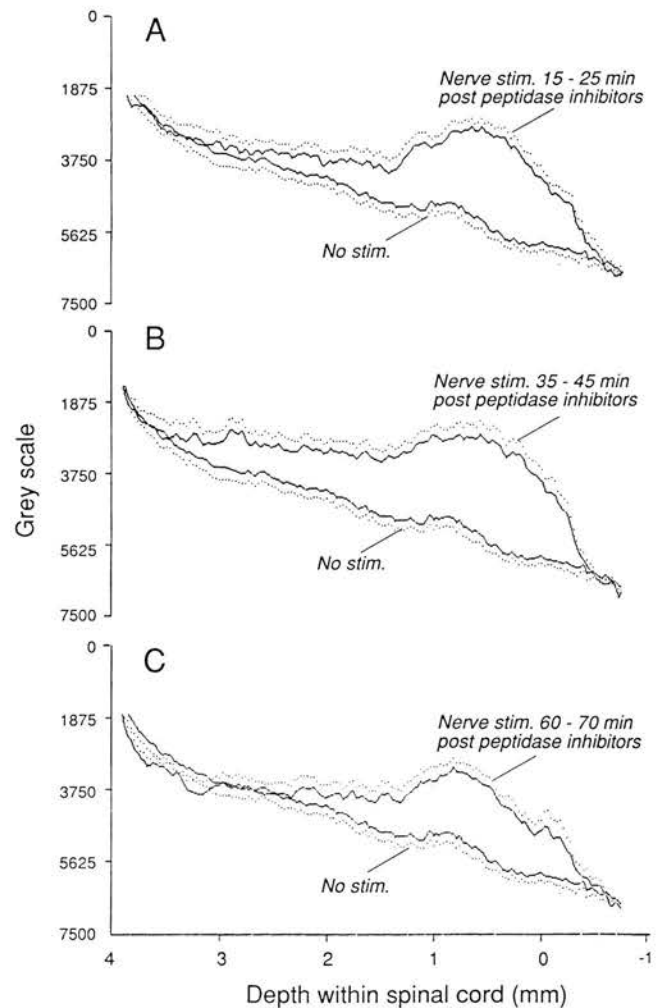


Fig. 2. Effect of peptidase inhibitors on the pattern of release of ir SP. The peptidase inhibitors ketorphan or ketorphan and enalaprilat were microinjected at depths of 2.5, 2.0, 1.5 and 1.0 mm prior to insertion of the microprobes displayed. Each graph shows the averaged image analysis of the 'No stim.' probes ($n = 19$, same group in all graphs) and the averaged image analysis of a group of microprobes present in the cord during electrical nerve stimulation in a specific period post-microinjection ($n = 15$ probes in A; $n = 15$ probes in B; $n = 13$ probes in C).

exhibited inhibition of binding of ^{125}I -SP from 0.5 mm to 2.0 mm from the dorsal surface with a peak at 0.8–1.2 mm (see also Fig. 3A). This deficit indicates prior binding of ir SP to the probes in this area of the spinal cord.

In confirmation of previous results⁹, microinjection of Ringer solution in volumes similar to those used with peptidase inhibitors did not alter stimulus-evoked release of ir SP in the dorsal horn. Fig. 1B compares the mean image analysis of 14 microprobes inserted in the spinal cord in the periods 15–25 and 35–45 min after microinjection of Ringer solution into the dorsal horn and during peripheral nerve stimulation with the corresponding pre-injection microprobes. There are no significant differences between these groups.

Microinjection of peptidase inhibitors

When peptidase inhibitors were microinjected into the grey matter the probes present in the cord during nerve

stimulation showed a significantly different pattern of ^{125}I -SP binding which progressed with time after injection. Fig. 2A displays the averaged image of 15 microprobes which were in the spinal cord in the period 15–25 min post-microinjection of peptidase inhibitors, either kelatorphan alone or kelatorphan and enalaprilat, at depths of 2.5, 2, 1.5 and 1 mm. Peripheral nerve stimulation was used with all. These probes show a zone of reduced grey density extending from the surface of the cord down to 2.0 mm, again with a peak at about 1 mm. At 35–45 min after microinjection of the peptidase inhibitors the zone of reduced binding of ^{125}I -SP had extended even further (Fig. 2B) down to 3.5 mm from the dorsal surface of the spinal cord. Fig. 2C shows a partial recovery from the effects of the peptidase inhibitors at the period 60–70 min after their microinjection.

The relation of such reduced binding of [^{125}I]SP to the anatomy of the spinal cord is shown in Fig. 3. In Fig. 3A a plot of t -values (30 μm separation) derived from

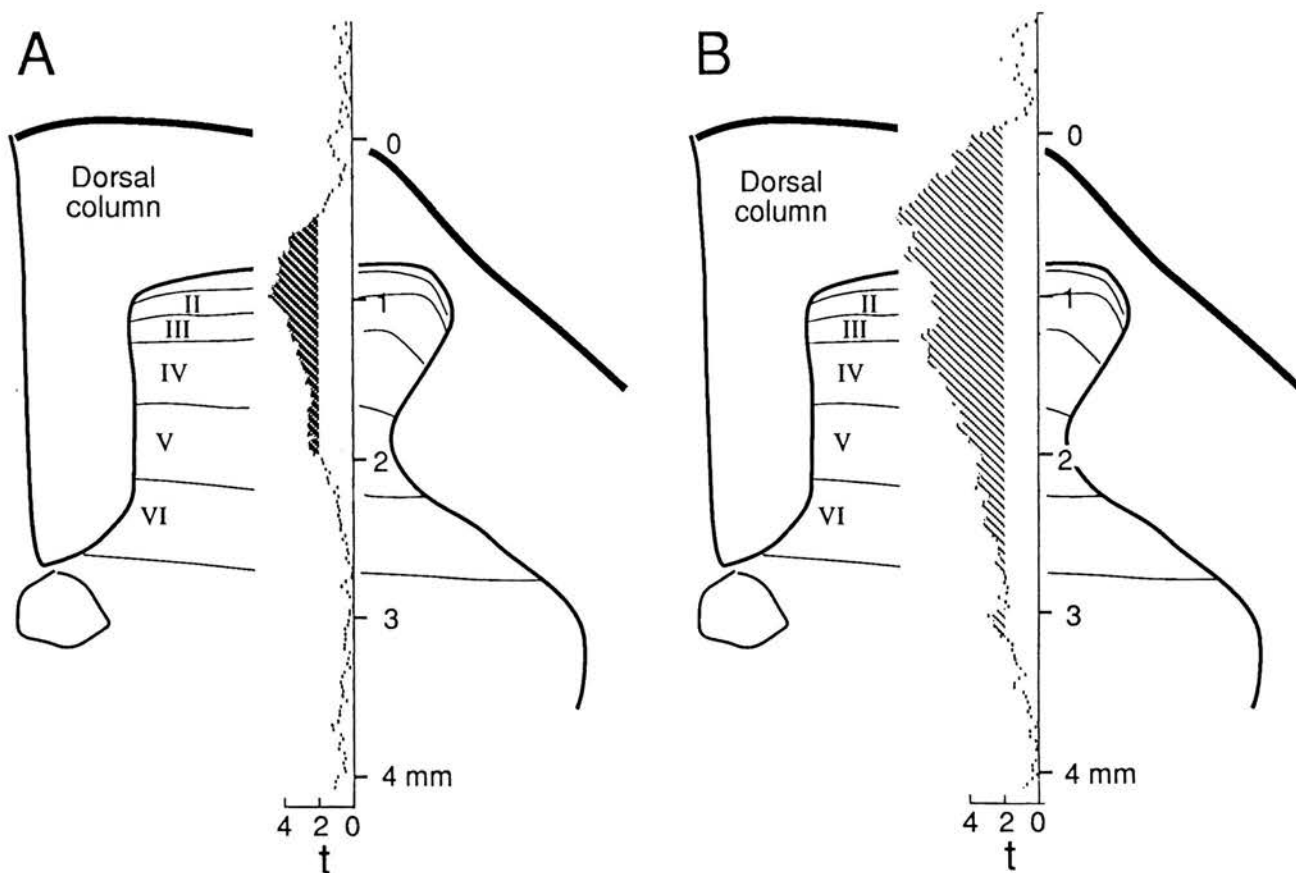


Fig. 3. The spinal laminar distribution of nerve stimulus-evoked release of ir SP before and after microinjection of peptidase inhibitors. A: a plot of the t -statistics derived from the differences between the averaged image of the 'No stim.' microprobes and the averaged image of the 'Nerve stim.' microprobes shown in Fig. 1A. B: a corresponding plot of the differences between the images of the 'No stim.' microprobes and the 'Nerve stim. post-peptidase inhibitors' microprobes shown in Fig. 2B. The significances were calculated in 30 μm intervals. 2T corresponds to $P < 0.05$ and 4T to $P < 0.01$. The shaded areas indicate sites in the cord in which the 'stimulus curves' were significantly different from the non-stimulation baseline, with $P < 0.05$. The ordinate in each plot is distance along microprobes in mm and this has been superimposed on an appropriately scaled diagram of a cross section of the lumbar spinal cord of the cat. The Roman numerals indicate the laminae of Rexed.

the differences of the means of the two image analyses of Fig. 1 are related to spinal laminae. In Fig. 3B the t -values derived from the differences of means of the image analyses of Fig. 2B are similarly plotted. These figures compare the effects of nerve stimulation before and after injection of peptidase inhibitors. They show that the zone of nerve stimulus-evoked presence of ir SP expanded both dorsally and ventrally following microinjection of peptidase inhibitors. Fig. 3B shows that the greatest effect of the stimulus was to elevate levels of ir SP in the dorsal columns. The no-stimulus group of Figs. 1A and 2B, however, has a small but non-significant zone of inhibition of binding of ^{125}I SP in laminae I and II and it is this which results in the greatest change produced by nerve stimulation post-peptidase inhibition to be seen in the dorsal column.

This shift is further apparent in Fig. 4 which plots the t -values derived by subtracting the mean image analysis

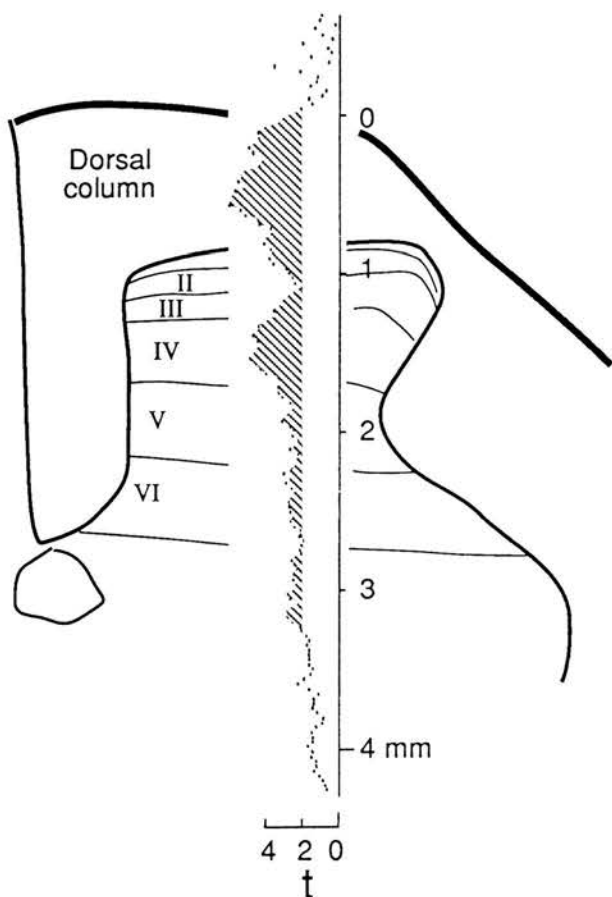


Fig. 4. The spinal distribution of the changes in nerve stimulus-evoked release of ir SP produced by microinjection of peptidase inhibitors. This is a plot of the t -statistics derived from the differences of means of the two mean image analyses shown in Fig. 2B ($n = 15$, 35–45 min after peptidase inhibitors and during nerve stimulation) and Fig. 1B ($n = 14$, after Ringer solution microinjection and during nerve stimulation). The hatched area highlights where $t > 2$ and where significance occurs at $P < 0.05$. The Roman numerals indicate the laminae of Rexed.

of microprobes inserted after microinjection of Ringer solution from those of microprobes present 35–45 min after microinjection of peptidase inhibitors. With both groups the ipsilateral tibial nerve was electrically stimulated. Although the peptidase inhibitors did produce a significant increase of the stimulus-evoked release of ir SP in the region of lamina II (at about 1 mm), the change in the levels of ir SP was significantly higher in the adjacent dorsal column and lamina IV. This results from the normal low levels in these latter areas following peripheral nerve stimulation.

Kelatorphan vs. kelatorphan and enalaprilat

Separate analysis of the probes present in the cord post-microinjection of kelatorphan and those inserted post-microinjection of both peptidase inhibitors showed that the groups were essentially similar at all periods. Fig. 5 shows the overlap of the averaged images of the two groups of probes both of which were in the cord from 35–45 min post-microinjection ($n = 7$ probes post-kelatorphan and $n = 8$ probes post-kelatorphan plus enalaprilat). The values derived from the differences between these two analyses do not show significant differences.

Effects of microinjection of peptidase inhibitors restricted to laminae I and II

In the experiments described above peptidase inhibitors had been microinjected at depths of 2.5, 2.0, 1.5 and 1.0 mm, in order to inhibit peptidases throughout the grey substance over a wide area of the dorsal horn. In a smaller number of experiments the microinjection was restricted to the region of laminae I and II, i.e. to a

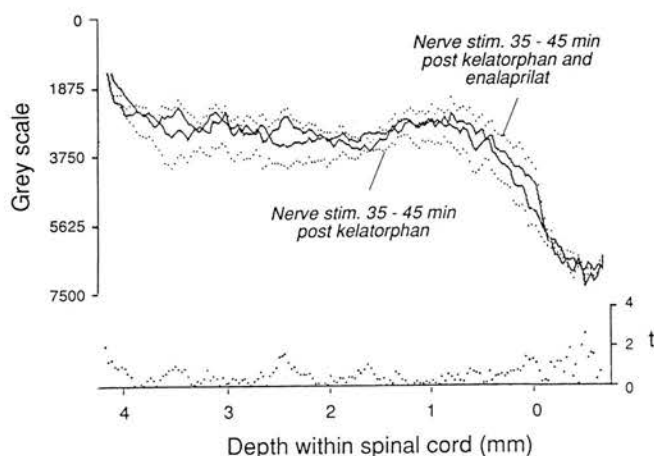


Fig. 5. A comparison of groups of microprobes inserted during electrical stimulation of the tibial nerve, 35–45 min post-microinjection of kelatorphan ($n = 7$ probes) and of kelatorphan plus enalaprilat ($n = 8$ probes), at depths of 2.5, 2.0, 1.5 and 1.0 mm from the dorsal surface of the spinal cord. A plot of the t -statistics derived from the differences of the means of the two groups is shown below.

depth of 1 mm. In these experiments the protocol was different in that the probes were kept in the cord for periods of 20 min and the stimuli were applied for longer times (electrical nerve stimulation) or repeated more often (noxious pinch). Fig. 6 shows that the restricted microinjection of peptidase inhibitors at a depth of 1 mm was followed by an expansion of the region in which stimulus-associated ir SP was detected.

Effect of peptidase inhibitors on the pattern of ir-SP evoked by noxious mechanical stimulation

Since electrical nerve stimulation is a quantitatively well-defined but unphysiological stimulus, we investigated whether the effect of peptidase inhibitors could also be demonstrated with ir SP released by natural stimuli. Fig. 7 displays the result of these experiments. The stimulus was noxious pinch to the toes of the ipsilateral hind paw, using crocodile clips, applied twice for 3 min in a 10 min test period.

Fig. 7A shows the release of ir SP evoked by pinch

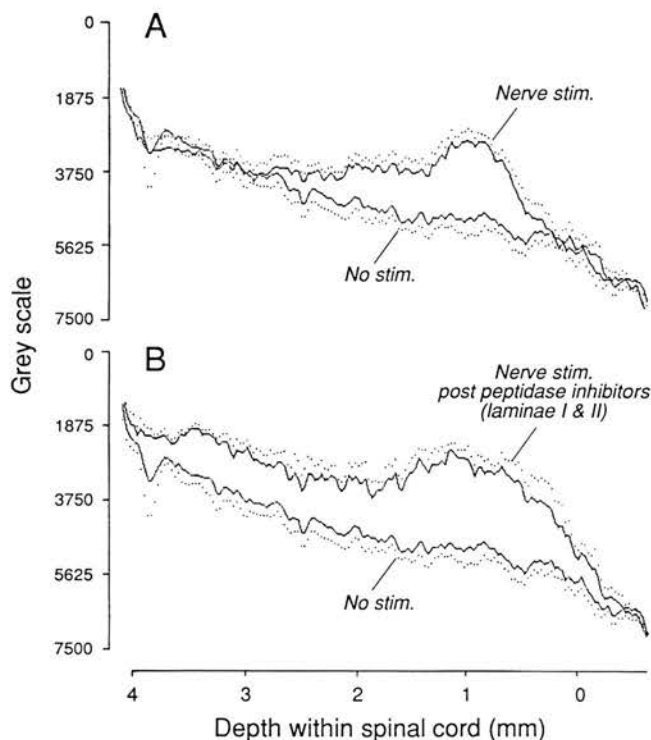


Fig. 6. Effect of the microinjection of peptidase inhibitors in the region of laminae I and II on the pattern of release of ir SP. All probes were in the spinal cord for 20 min. A: release of ir SP by electrical stimulation of the tibial nerve (20 Hz, 50 V, 0.5 ms pulse duration). 'No stim.' group: $n = 5$ probes; 'Nerve stim.' group: $n = 13$ probes. These control microprobes are restricted to those experiments in which peptidase inhibitors were only microinjected in the region of laminae I and II. B: nerve stimulus-evoked release of ir SP 55–75 min following microinjection of kelatorphan and enalaprilat at 1.0 mm from the dorsal surface of the spinal cord. 'Nerve stim. post-peptidase inhibitors (laminae I & II)' group: $n = 4$. The 'No stim.' group is the same as that shown in A.

prior to injection of peptidase inhibitors. The mean image analysis of 21 probes present in the cord in the absence of peripheral stimulation is compared with that of 46 probes present in the cord during the application of noxious pinch. The stimulus produced increased levels of ir SP in a restricted area of the superficial dorsal horn. Following microinjection of peptidase inhibitors at depths of 1.0 to 2.5 mm from the dorsal surface, this zone progressively expanded with peripheral nerve stimulation (Fig. 7B and C). At 50–60 min after peptidase inhibitor microinjection, noxious pinch produced significantly increased levels of ir SP over the whole of the dorsal horn and dorsal columns.

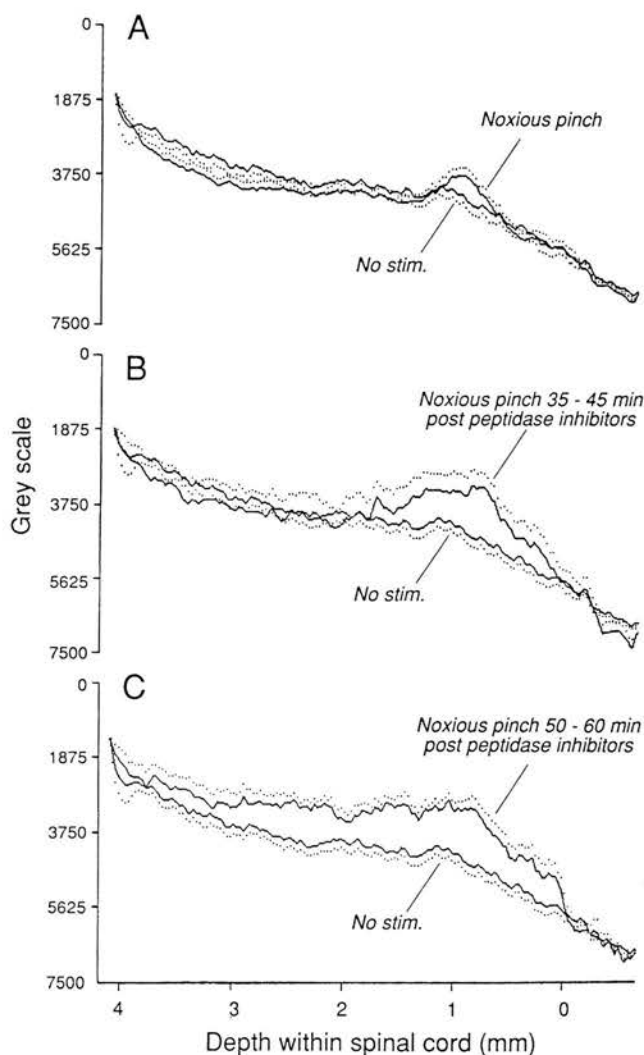


Fig. 7. Effect of the microinjection of peptidase inhibitors at 2.5, 2.0, 1.5 and 1.0 mm from the dorsal surface of the spinal cord on the pattern of release of ir SP evoked by noxious mechanical stimulation. Each graph shows the averaged image of the 'No stim.'-probes of the control period ($n = 21$) and the averaged image of probes present in the cord during noxious pinch under the conditions indicated ($n = 46, 15$ and 11 from top to bottom). Each probe was in the cord for 10 min; noxious pinch of the toes was applied twice for 3 min in the 10 min period.

DISCUSSION

The present results have shown that microinjection of peptidase inhibitors into the dorsal horn of the spinal cord results in a remarkably diffuse pattern of inhibition of binding of ^{125}I -SP to the immobilised antibodies of microprobes. Prior to discussing the physiological significance of this finding, some technical considerations require examination.

In vitro tests showed that high concentrations of kelatorphan and enalaprilat did not inhibit the binding of ^{125}I -SP to microprobes. Hence, the present results cannot have resulted from a diffuse binding of these compounds to microprobes following microinjection into the spinal cord. The simplest explanation of the altered binding curves produced by the peptidase inhibitors is that this resulted from increased binding of ir SP to microprobes associated with increased levels at many sites in the dorsal horn. As previously discussed^{5,6} a localised inhibition of binding of an exogenous ligand could also result from damage to antibodies from proteases either present in the extracellular fluid of the spinal cord or liberated from damaged cells. Such a proposal also requires, however, that proteases be released centrally following certain peripheral stimuli to explain the increased inhibition of binding shown on microprobes following such stimuli. The different patterns of inhibition of binding produced by identical peripheral stimuli on microprobes bearing different antibodies, however, is very difficult to reconcile with such a hypothesis. It is equally difficult to explain wide-spread inhibition of the binding of ^{125}I -SP to microprobes as resulting from enhanced activity of proteases following microinjection of compounds which inhibit peptidases. Thus the present experiments provide important support for the validity of results obtained with the antibody microprobe technique.

Within the spinal grey matter a pattern of widespread presence could represent diffusion of ir SP away from sites of release or simply a revealing of minimally released ir SP protected from degradation at sites of release. The greatest change in the levels of ir SP detected following peptidase inhibition, however, occurred in the dorsal column white matter (Fig. 4) where there are virtually no nerve endings and, hence, no sites of release. This change must have resulted from diffusion of protected ir SP from sites of release in the superficial dorsal horn. It is probable, therefore, that the pattern of release of ir SP observed in the spinal grey matter following peptidase inhibition predominantly results from diffusion away from the sites of release detected in the normal spinal cord.

The present experiments indicate that enzymic degradation is an important determinant of the degradation of

substance P under physiological conditions in the spinal cord. Since kelatorphan inhibits aminopeptidases, depeptidylaminopeptidases and endopeptidase 24:11 (ref. 32) it is not possible to state which is the more important physiologically. Kelatorphan alone produced effects similar to the combined administration of kelatorphan and enalaprilat and, hence, it is probable that angiotensin-converting enzyme does not contribute significantly to the degradation of substance P at least in the spinal cord in vivo. In slice preparations from the substantia nigra of the rat²² and the dorsal spinal cord of the guinea pig¹² the converting enzyme inhibitor captopril failed to modify the levels of ir SP released in response to either high external K^+ (substantia nigra) or capsaicin added to the perfusate (spinal cord) supporting the conclusion of the present study. Both of these studies found that inhibitors of endopeptidase 24.11 (enkephalinase) such as phosphoramidon, thiorphan and kelatorphan caused increased release from the slice preparation following either high external K^+ or capsaicin.

One difficulty in the interpretation of such experiments is that the enzymes inhibited may be important in metabolising a number of neuropeptides in addition to substance P. Kelatorphan potentiates the actions of met-enkephalin administered microiontophoretically in the substantia gelatinosa of the cat²⁶ and applied to the surface of the rat spinal cord⁴. It also increases high K^+ evoked release of met-enkephalin from spinal cord slices²⁴. All of these actions probably result from inhibition of endopeptidase 24:11. Opioids have been shown to reduced high K^+ -evoked release of SP from a slice preparation¹⁷ and, as a result, proposed as exerting a presynaptic inhibitory control of the release of SP from the central terminals of nociceptors¹⁷. Mauborgne et al.²⁴ proposed that a potentiation of such a control was responsible for their failure to observe an enhancement, by kelatorphan, of high K^+ -evoked release of SP from a rat spinal cord slice. Such a result was anticipated in the design of the present experiments, since a potentiated block of SP release by protected opioid peptides could effectively prevent the detection of any impaired degradation of released SP. That the present experiments readily found evidence of enhanced levels of ir SP following the microinjection of peptidase inhibitors does suggest, however, that tonic control of SP release from the central terminals of nociceptors is not a significant factor in the anaesthetized cat. It needs to be pointed out moreover that experiments suggesting such a control have come from experiments on slice preparations, where elevated K^+ was used as a stimulus for release of SP and these have the problem of not knowing the source of SP. High K^+ will depolarize virtually all neural elements in a slice and it is just as likely that the depression of such

release by opioids is exerted postsynaptically on intrinsic neurons of the slice as on the severed central terminals of nociceptors.

The most significant result of the present experiments is the demonstration that enzyme inhibition not only determines the levels of substance P sustained at sites of release but also the sites accessed following release. The microprobe method is particularly suited to study spread following release, since it samples continuously along its length. In essence, the present experiments have shown that peptidase inhibition causes the pattern of presence of ir SP following stimulus-evoked release to resemble that of ir NKA^{8,15}.

In vitro, NKA is relatively resistant to degradation by plasma enzymes³⁰ and by an endopeptidase degrading SP isolated by Nyberg et al²⁷. Indeed it has been emphasised that the potency of tachykinins in some in vitro preparations is not only determined by the relative abundance of NK₁, NK₂ and NK₃ receptors but also by the activity of degrading enzymes²⁹.

The first convincing demonstration that a synaptically released neuropeptide could act at a distance was that of Jan and Jan¹⁶ on neurones of bull frog sympathetic ganglia. Thus, electrical stimulation of a preganglionic nerve produced a depolarisation (mimicked by application of a luteinising hormone releasing hormone, LHRH) in neurones not receiving an innervation by LHRH containing nerve endings. Within the central nervous system this concept of an action distant from a site of release has been termed volume transmission^{1,11}. It has been proposed¹¹ that transmission at a synaptic site of release is characterised by low-affinity receptors (concentrations are high) whereas effects distally are mediated by high-affinity receptors which may also be activated by degradation products as well as the original neuropeptide. The present results are supportive of these proposals, since

they show that prolonging the life of a synaptically released neuropeptide results in diffusion to many sites outside sites of release, a situation previously observed with ir NKA.

It appears, therefore, that the transmission of information from nociceptive primary afferents has several components. There is evidence that L-glutamate is contained within and released from¹⁹ the central terminals of small diameter primary afferents. L-Glutamate is rapidly removed from synapses¹⁸ and excitation at AMPA receptors for L-glutamate is probably a major instance of 'fast' transmission within the central nervous system. At the other extreme may be the long lasting, non-localised effects of ir NKA. Probably supporting this latter proposal is the recent report that the prolonged (greater than 1 h) facilitation of a flexor reflex in the decerebrate rat produced by brief electrical stimulation of unmyelinated primary afferents of the nerve to the gastrocnemius-soleus muscles was blocked by an antagonist acting at NK₂ receptors, which are high affinity receptors for NKA³³. The situation is potentially plastic, since there is evidence that the enzymatic degradation of substance P (and probably NKA) is regulated by calcitonin gene-related peptide, a compound probably co-released with tachykinins. The functional significance of somatopically diffuse effects of compounds released from nociceptors is presently obscure but they could act to produce widespread alterations of normal function resulting in immobilising or guarding a discrete source of injury.

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Persistence of immunoreactive neurokinins in the dorsal horn of barbiturate anaesthetized and spinal cats, following release by tibial nerve stimulation

P.J. Hope, C.W. Lang and A.W. Duggan

Department of Preclinical Veterinary Sciences, University of Edinburgh (U.K.)

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Key words: Antibody microprobes; Neurokinin release; Spinal cord; Peripheral nerve stimulation

Antibody-coated microprobes were used to study the time course of release and disappearance of immunoreactive neurokinins in the dorsal spinal cord, in response to electrical stimulation of unmyelinated fibres of the tibial nerve of the cat. Noxious cutaneous stimuli were not used thereby avoiding potentially uncontrolled tissue damage and inflammation. Microprobes, inserted into the spinal cords of barbiturated anaesthetized spinal cats prior to nerve stimulation, detected a basal level of immunoreactive neurokinins. During nerve stimulation immunoreactive neurokinins were released significantly in the upper dorsal horn and dorsal columns and required at least 1 h to return to prestimulus levels. The persistence of immunoreactive neurokinins in the dorsal horn may underlie the prolonged hyperexcitability of some spinal neurons following brief noxious stimuli.

The antibody microprobe is a device well suited to localize sites of release of neuropeptides within the central nervous system [5, 6]. Thus in experiments employing microprobes coated with antibodies to substance P (SP), spinal immunoreactive (ir) SP release evoked by noxious cutaneous stimuli, was centered on the substantia gelatinosa of the dorsal horn with a second, deeper, area approximating to laminae V and VI [6]. Such restricted areas of release were not seen in experiments using similar noxious cutaneous stimuli, but employing microprobes bearing antibodies to neurokinin A (NKA). Noxious stimulus-evoked release of irNKA was detected widely in the dorsal spinal cord of the cat, including the overlying dorsal columns [7]. This was an unexpected result since immunohistochemical studies indicate that these neuropeptides have a similar distribution within the upper dorsal horn of the rat [4].

In addition there was evidence that irNKA persisted beyond the duration of a noxious stimulus. Since there is evidence that NKA is resistant to degradation by processes which rapidly inactivate SP [16, 18] it was proposed that the apparent persistence and diffusion of irNKA away from sites of release from nerve terminals, most probably resulted from a natural slow inactivation of this compound. This conclusion has important impli-

cations for the possible functions of NKA in the spinal cord, particularly in view of the several reports that prolonged hyperexcitability of spinal neurones can follow a relatively brief input from nociceptors [2, 3, 9, 11, 15, 19, 20].

When using noxious stimuli applied to the skin, there is always the possibility that tissue damage is produced by a particular stimulus and hence that nociceptors will continue to fire, at a reduced rate, after the period of stimulus application. Thus the persistence of irNKA in the experiments cited could have resulted from irNKA being released from a subset of nociceptors activated by minimal tissue damage. This possibility can be investigated by employing electrical stimulation of a peripheral nerve as the stimulus to evoke neuropeptide release, since there is no evidence that afferent fibres fire for periods beyond the application of an electrical stimulus. Thus the present experiments have used antibody microprobes to study spinal release of ir neurokinins produced by electrical stimulation of the tibial nerve in the anaesthetized spinal cat.

Experiments were performed on 8 cats anaesthetized with sodium pentobarbitone (35 mg/kg, i.p. initially and maintained with an intravenous infusion of 3–6 mg/kg/h). Blood pressure and end tidal CO₂ levels were monitored continuously. Five cats were paralysed with intermittent (90 min) injections of gallamine triethiodide (4 mg/kg) and artificially ventilated. The spinal cord was exposed by removal of the lumbar laminae and tran-

Correspondence: A.W. Duggan, Department of Preclinical, Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh, EH9 1QH, U.K.

sected at the thoraco-lumbar junction after injection of local anaesthetic at the site of section. The dura was opened with sterile scissors and then covered with sterile agar. The agar was removed locally to allow microprobe penetration and small areas of pia-arachnoid were removed with sterile fine forceps. These exposed areas were irrigated continuously with a sterile Ringer solution at 37°C and removed by suction. It is important to prevent contamination and infection of the spinal cord, in experiments which measure neuropeptide release, since the latter are contained in inflammatory exudates. The animal also received two subcutaneous injections of an ampicillin suspension (25 mg/kg). The left and right tibial nerves were exposed in the hind limbs, mounted on stimulating electrodes and immersed in liquid paraffin pools to prevent dehydration.

Antibodies were immobilized onto the outer surface of glass micropipettes as previously described by Duggan et al. [5]. The antisera used were from two sources – Peninsula Laboratories and Milab. Both have complete cross-

reactivity with neuropeptide K, an N-terminal extended neurokinin A, 70–80% cross-reactivity with neurokinin B, but negligible cross-reactivity with substance P. For the purposes of the present experiments microprobes are described as detecting immunoreactive neurokinins (ir NK).

Microprobes were filled with 2% Pontamine sky blue in 0.2 M sodium acetate and then introduced 3 mm into the spinal cord, in pairs, via independently controlled stepping-motor micromanipulators. Initially, field potentials in response to electrical stimulation of the ipsilateral tibial nerve (A fibre strength) were recorded and an optimal distance from the mid-line was chosen (on the basis of these recordings) for the subsequent introduction of microprobes. The threshold stimulus for production of field potentials using pulses 0.3 ms duration were determined at this time. A typical protocol consisted of 3–4 pairs of microprobes remaining in the spinal cord for 15–30 min in the absence of any stimulus, followed by two comparable periods of ipsilateral

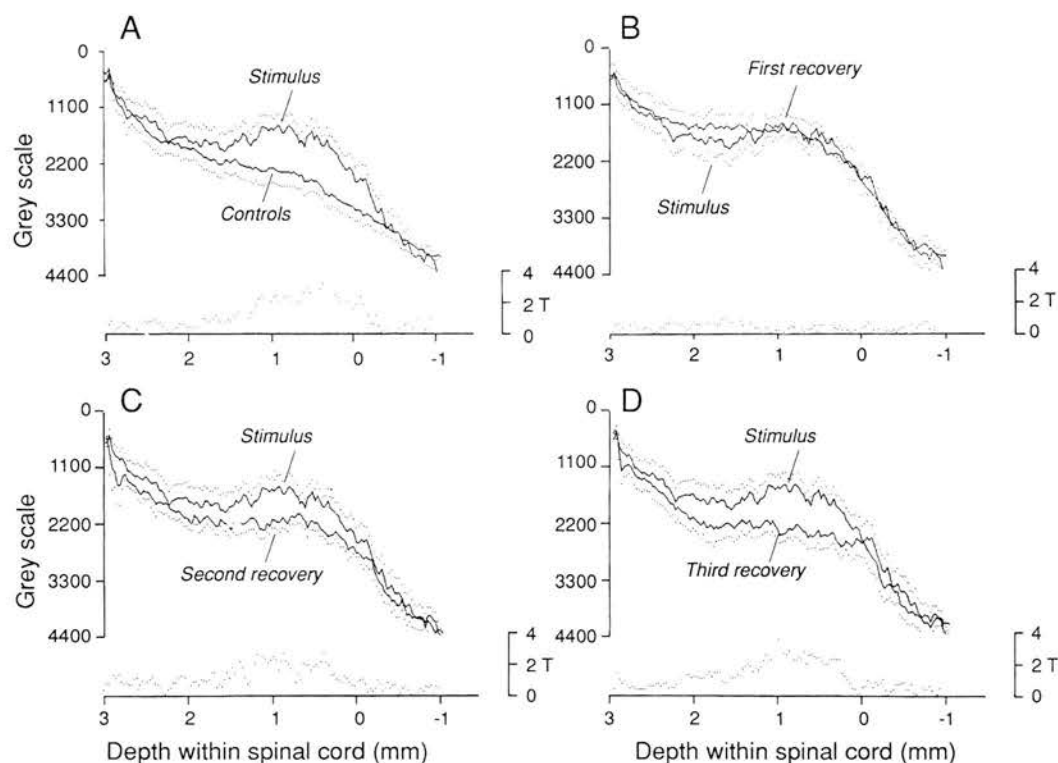


Fig. 1. Comparisons of the levels of immunoreactive neurokinins in the dorsal horn before, during and at various times after peripheral nerve stimulation. The mean image analyses (\pm S.E.M.) of 5 groups of microprobes bearing antibodies to neurokinins are shown. All microprobes were inserted in the spinal cord to a depth of 3 mm, for a duration of 30 min. A, B, and C (upper panels): in each section a graph described as *stimulus* is the mean image analysis of microprobes ($n=11$) inserted in the spinal cord whilst the ipsilateral tibial nerve was stimulated at an intensity sufficient to excite C-fibres (continuous tetanus of 0.3 ms pulses, at 10 Hz, $\times 100$ threshold). In each of the upper panels this graph is compared with another described as: A: *controls*. Microprobes inserted into the cord immediately prior to any nerve stimulation ($n=18$). B: *first recovery*. Microprobes inserted into the cord immediately after cessation of nerve stimulation ($n=7$). C: *second recovery*. Microprobes inserted into the cord for the period 31–60 min after cessation of nerve stimulation ($n=11$). D: *third recovery*. Microprobes inserted into the cord for the period 61–90 min after cessation of nerve stimulation ($n=12$). A, B, C and D (lower panels): plots of the T values ($2T = P < 0.05$) derived from the differences between the two graphs of each upper panel.

electrical nerve stimulation (2 pairs of microprobes). A further 4 pairs of microprobes were then introduced over the next 2 h, each remaining 15–30 min in the spinal cord. After an interval of 1–2 h, this sequence was repeated on the other side of the spinal cord whilst stimulating the opposite tibial nerve. The electrical stimulus was a continuous tetanus (10 Hz) of 0.3 ms pulses, the stimulus strength being $\times 100$ threshold for production of a spinal cord field potential.

As previously described [5, 7], after withdrawal from the spinal cord, microprobes were washed and incubated in ^{125}I -NKA. Autoradiographic images of the microprobes were then obtained by placing their tips on monoemulsion X-ray film (Kodak, NMC) [5]. These images were analysed using the method of Hendry et al. [8] using a computerized image analysis system to measure optical density in 10 μm steps along the length of each autoradiographic image. For clarity, in the illustration of this paper the means of 3 successive sites (30 μm) are shown.

The experiments employed 94 microprobes inserted into the spinal cord. In addition, in each experiment 4–6 microprobes were also used for concurrent *in vitro* tests of microprobe sensitivity.

As found previously [7], microprobes inserted into the spinal cord in the absence of any peripheral stimulus, and prior to any noxious stimulus, showed a widespread basal inhibition of binding of ^{125}I -NKA when compared with *in vitro* microprobes, not exposed to NKA, but simply incubated in the labelled peptide.

The basal levels of ir NK were increased by electrical stimulation of A and C fibres of the ipsilateral tibial nerve. This effect is shown for microprobes remaining in the spinal cord for 30 min in Fig. 1A.

As observed previously [7] this increase in ir NK occurred relatively diffusely over the upper dorsal horn including the dorsal columns. Within the first 30 min after cessation of nerve stimulation the levels of ir NK detected in the dorsal horn did not significantly decline (Fig. 1B). Indeed in the deep dorsal horn the mean image analysis of microprobes in this first recovery period is displaced above that of microprobes present during the stimulus, suggesting diffusion away from the sites of release, but these differences are not significant. In the period 31–60 min after nerve stimulation (second recovery) the mean image analysis of microprobes (Fig. 1C) is uniformly below that of those within the spinal cord during nerve stimulation, but the differences attain significance only in limited areas. At 61–90 min after nerve stimulation (third recovery) however (Fig. 1D) recovery is virtually complete, as shown by the plot of the significance of differences between these microprobes and those in the spinal cord during nerve stimulation (Fig.

1D, lower panel).

The present experiments have shown ir NK released by peripheral nerve stimulation persists in the dorsal horn in elevated amounts for at least an hour beyond the cessation of the stimulus. This persistence probably accounts for the diffuse presence of ir NK detected by microprobes when compared with the localized presence of substance P [6] calcitonin gene-related peptide [13] and somatostatin [14] in the region of the substantia gelatinosa.

Neuropeptides appear to be degraded mainly by enzymes but there are often difficulties in determining which enzymes are responsible for the major part of degradation of a particular peptide under physiological conditions and whether these enzymes are located adjacent to sites of release. Whilst a number of enzymes degrade SP [1, 16], of these only endopeptidase 24.11 significantly degrades NKA, and at a slower rate [10, 18]. Thus there are reasonable biochemical grounds to support the conclusion of the present experiments that neurokinins, particularly NKA, may be slowly degraded when released in the spinal cord.

Although some reviews have drawn attention to the failure of peptidases to be located adjacent to presumed sites of release of neuropeptides [1, 12], there is little information on this question when considering tachykinins in the spinal cord. In an autoradiographic study of the distribution of endopeptidase 24:11, relatively high levels of this enzyme were found in the substantia gelatinosa of the spinal cord, but there were much higher levels in the overlying pia and dura in both the rat and man [21]. It was suggested that meningeal enzymes protect the brain and spinal cord from neuropeptides in the cerebrospinal fluid (CSF) but the present studies suggest the reverse: meningeal enzymes may prevent the escape of spinally released neuropeptides into the subdural space.

In the present study the immunoreactivity detected by microprobes cannot be identified in exact molecular terms but is most likely neurokinin A or neuropeptide K. A contribution by neurokinin B cannot be excluded. Although this latter compound is not present in significant amounts in dorsal roots [17] it is within neurones of the upper dorsal horn which could have been activated by the stimuli used in the present experiments. Differentiation between these compounds requires the use of antibodies directed at non-homologous regions of these peptides.

The persistence and diffusion of ir NK suggests a role different from that of a transmitter conveying information about the onset, location and duration of a peripheral noxious stimulus. As cited previously, however, several laboratories have found prolonged hyperexcitability of some spinal neurones following a brief input

from nociceptors [2, 3, 9, 11, 15, 19, 20] and neurokinins warrant consideration as being involved in these changes.

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Sustained isometric contraction of skeletal muscle results in release of immunoreactive neurokinins in the spinal cord of the anaesthetized cat

A.W. Duggan¹, P.J. Hope¹, C.W. Lang¹ and C.A. Williams²

¹Department of Preclinical Veterinary Sciences, University of Edinburgh, Edinburgh (U.K.) and ²Department of Physiology, Quillen College of Medicine, East Tennessee State University, Johnson City, TN (U.S.A.)

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Antibody microprobes were used to study release of immunoreactive neurokinins in the dorsal horn of the anaesthetized spinal cat following sustained isometric contraction of ipsilateral hindlimb muscles. Microprobes had immobilized antibodies to neurokinin A (NKA) on their outer surfaces and bound a proportion of released molecules when inserted in the central nervous system. Bound molecules were detected in autoradiographs as zones of reduced binding of ¹²⁵I-NKA in which microprobes were incubated after withdrawal from the spinal cord. The left hindlimb was immobilized using an epoxy bandage splint and isometric contraction of muscles induced by intermittent tetanic stimulation of a ventral root. A basal presence of immunoreactive neurokinins was detected and this was increased by sustained isometric muscle contraction. It is probable that ergoreceptors contain and release neurokinins.

A variety of spinal processes are activated by an input from skeletal muscle afferents. In addition to the fast transmission from Group I and II afferents to motoneurons and cells of origin of ascending tracts, there is evidence for longer lasting events. Thus electrical stimulation of small diameter myelinated and unmyelinated afferents of the nerve to the gastrocnemius-soleus muscle of the rat has been shown to produce long lasting facilitation of a flexor reflex elicited by intermittent stimulation of the sural nerve [14]. In addition, isometric contraction of skeletal muscle is associated with circulatory and respiratory changes believed to be important in exercise [2, 11, 12]. These latter changes are dependent on impulses in muscle afferents, since they are abolished by section of the dorsal roots containing afferents from the muscle being stimulated. Experiments using anodal block of impulse propagation in large fibres and also local anaesthetic injections into dorsal roots suggested that small diameter afferents were important [12]. The relevant afferents which are activated by muscle stretch, pressure or metabolites have been termed ergoreceptors and they include both small myelinated and unmyelinated fibres. Ergoreceptors have been differentiated

from nociceptors, although a small proportion of the latter can fire with muscle contraction under ischaemic conditions [10].

Our interest in this area derives from experiments using antibody microprobes to localize sites of release of tachykinins within the spinal cord following either electrical stimulation of peripheral nerves or natural stimuli applied to skin or joints [5, 6, 8]. Both immunoreactive substance P (irSP) and ir neurokinin A (irNKA) were released in the spinal cord by electrical stimulation of unmyelinated primary afferents. When using natural stimuli however it appeared that spinal release of irSP was best seen with damaging stimuli to the skin or when inflammation was well developed in a joint. Release of irNKA was observed with noxious but not necessarily damaging skin stimuli and immediately when a joint was injected with kaolin and carrageenan. In addition ir NKA persisted in the spinal cord for in excess of 30 min after a period of adequate stimulation [8].

It is of importance to determine if activation of ergoreceptors results in a spinal release of tachykinins. Since previous results indicated that irNKA rather than irSP was better associated with subdamaging stimuli, the present experiments have studied the spinal release of irNKA during sustained isometric contraction of hindlimb muscles of barbiturate-anaesthetized cats.

Experiments were performed on 6 cats anaesthetized with pentobarbitone (35 mg/kg, i.p., initially and main-

Correspondence: A.W. Duggan, Department of Preclinical Veterinary Sciences, University of Edinburgh, R.(D.)S.V.S., Summerhall, Edinburgh EH9 1QH, U.K.

tained with an intravenous infusion of 3–6 mg/kg/h). Arterial blood pressure and end tidal CO₂ levels were monitored continuously. All animals were artificially ventilated with air. The spinal cord was exposed by removal of the lumbar laminae. The dura was opened with sterile scissors and then covered with sterile agar. The agar was removed locally at proposed sites of microprobe penetration and within the exposed areas, small areas of pia-arachnoid were removed with sterile fine forceps for microprobe entry. These exposed areas were irrigated continuously with a sterile Ringer solution which was removed by suction. The fine polyethylene tubing delivering and removing the perfusate had also been sterilized. It is important to prevent contamination and infection of the spinal cord in experiments which measure the release of neuropeptides since the latter are contained in inflammatory exudates. The animal also received two injections of an ampicillin suspension, each of 25 mg/kg.

Sustained isometric contraction of lower leg muscles can be achieved by immobilizing the limb with pins driven through the joints and stimulating the appropriate nerves electrically [12]. For the present experiments it was felt that limb immobilization produced surgically could result in a large release of irNKA which might obscure any possible release evoked by sustained muscle contraction. Surgery to the hindlimb was therefore avoided by immobilizing the knee joint (leg extended) and ankle joint (held at approximately 90°) in an epoxy bandage splint (Vetcast 3M). A clamp then immobilized the bandage splint. Prior to covering the spinal cord with agar, the L₆, L₇ and S₁ ventral roots were exposed and sectioned close to their junction with the spinal cord. The ventral root giving a consistent maintained rise in blood pressure (see Fig. 1) when stimulated at 4 × threshold to elicit a muscle twitch (just visible above the proximal edge of the epoxy bandage) was used for subsequent test stimuli. Stimulus pulses were 0.1 ms in duration and a continuous tetanus was induced with repetitive stimulation of the central root at 40 Hz for 1 min and repeated at 5 min intervals while microprobes were in the spinal cord for 30 min (Fig. 1). Antibodies to NKA were immobilized to glass micropipettes through coating the latter successively with a polymer derived from aminopropyltriethoxysilane, glutaraldehyde, protein A and finally, antibodies [4]. The latter were derived from antisera purchased from Peninsula Labs. and Milab. Both had complete cross-reactivity with neuropeptide K, an N-terminal extended form of neurokinin A, 70–80% cross reactivity with neurokinin B but negligible cross-reactivity with substance P. For the purposes of the present experiments microprobes are described as detecting immunoreactive neurokinins

(irNKA). Prior to use in the spinal cord, the tips of microprobes were broken back to 5–10 µm in diameter and all were filled with a 1% (w/v) solution of Pontamine sky blue in 0.2 M sodium acetate. This enabled extracellular recordings to be obtained with microprobes as well as dye to be deposited from their tips for subsequent histological examination. All of the sites chosen for microprobe penetration were in the same spinal segment as that of the ventral root stimulated to produce isometric muscle contraction. Microprobes were introduced independently, two at a time, using stepping-motor micro-manipulators.

After withdrawal from the spinal cord, microprobes were washed for 15 min in cold PBS Tween and then incubated in ¹²⁵I-NKA for 24 h at 4°C. The concentration of labelled peptides for these incubations was 1.0 µCi/ml. After this incubation, microprobes were washed for 15 min in cold PBS Tween with suction applied to each microprobe to remove any contained labelled neuropeptide. The tips were then broken off and fixed to a sheet of paper and placed in a cassette with a sheet of monoemulsion X-ray film (Kodak NMC). The images of microprobes were analysed using the method of Hendry et al. [7]. Simply, this estimated optical density in 10 µm squares on a grey scale of 0–255. Transverse integrations were performed across each microprobe image to give a plot of optical density against length along a microprobe and hence of depth within the spinal cord. A search program subsequently computed a mean image analysis for defined groups of microprobes together with the standard errors of means. The method treats sites in the spinal cord progressively 10 µm from the dorsal surface, as independent. For clarity in the illustrations of this paper the means of 3 successive sites are shown.

Results were obtained using 79 microprobes. In addition in each experiment 4 to 6 microprobes were used for in vitro sensitivity tests. These showed that incubation in a solution containing 10⁻⁸ M NKA for 30 min at 37°C significantly reduced the binding of ¹²⁵I-NKA. Stimulation of a ventral root produced rises in systolic blood pressure of 20–40 mmHg as shown in Fig. 1B. This figure also shows that the effect was repeatable within the period (usually 30 min) microprobes were in the spinal cord. In the experiment illustrated the dorsal roots of segments L₅ to S₂ inclusive were subsequently sectioned and ventral root stimulation then failed to elicit a pressor response.

Previous experiments with microprobes bearing antibodies to NKA have found a remarkable persistence of irNKA beyond the duration of an effective peripheral noxious cutaneous stimulus [6, 8]. Thus when examining microprobes inserted into the spinal cord without any added stimulation (controls), it is necessary to differenti-

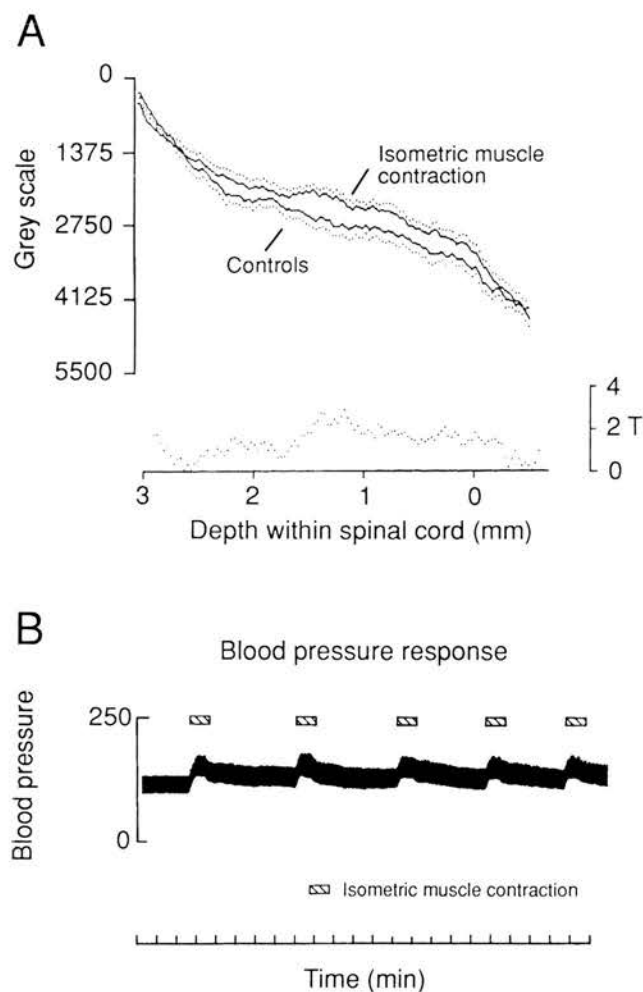


Fig. 1. Isometric muscle contraction and spinal release of irNK. A: Upper records. Mean image analyses of microprobes inserted for 30 minutes into the spinal cord in the absence of any stimulus and prior to periods of isometric muscle contraction ($n=15$) and those inserted for the same time but during isometric muscle contraction ($n=24$). The image analysis was performed in 30 μm steps. At each step the standard error of the mean image density is plotted below the mean for controls and above the mean for isometric muscle contraction. Lower record. A plot of the t -values for the differences between means in the upper records. B: an example of the rises in arterial blood pressure associated with the isometric muscle contraction following ventral root stimulation. Ordinate: mmHg.

ate between those inserted prior to any active stimulus and those inserted after a period of stimulation. Figure 1A illustrates the mean image analyses of control microprobes inserted for 30 minutes prior to ventral root stimulation ($n=15$) and, those inserted for the same time during ventral root stimulation ($n=24$, Fig. 1B).

Ventral root stimulation increased levels of irNK significantly in the superficial dorsal horn and adjacent dorsal columns, as shown by the plot of t -values for the differences between the two mean image analyses. As in previous experiments the effective stimulus resulted in detection of irNK over a relatively broad area. The mean

image analysis of 32 microprobes inserted 0–30 min after a period of ventral root stimulation showed persistence of increased levels relative to microprobes inserted prior to the stimulus. This persistence has also been observed with cutaneous stimuli and peripheral nerve stimulation. In the one experiment in which the dorsal roots were sectioned, this procedure produced a large release of irNK in the spinal cord.

The present experiments have shown spinal release of irNK following impulses in muscle afferents activated by isometric contraction. Although it is not possible to exclude the firing of some nociceptors under those conditions [10], it is probable that activity in ergoreceptors was responsible for the release of irNK. It is improbable that cutaneous nociceptors produced the observed release of irNK since the epoxy bandage immobilizing the limb was broadly applied and the fur of the cat acts as a natural padding. As in previous experiments, irNK was detected over a relatively broad area and extended into the dorsal columns [6, 8]. It has been previously proposed that this broad distribution resulted from a slow degradation of released molecules [6].

Small-diameter afferents from hindlimb muscles project mainly to the lamina I of the spinal cord [3] and release at this superficial site is probably responsible for the prominence of the diffusion of irNK into the dorsal columns. This location also favours the contention that release occurred mainly from the central terminals of primary afferents although the contribution from intrinsic spinal neurones cannot be assessed in experiments of this type.

Spinal release of a neurokinin may be a factor in the blood pressor response to isometric muscle contraction but previous experiments [6, 8] suggest that ir neurokinins persist beyond the duration of the blood pressor response as induced in the present experiments. Intrathecal administration of the tachykinin antagonist [D-Pro², D-Phe⁷, D-Trp⁹]-SP has attenuated the pressor response to isometric muscle contraction [9] but this compound does not readily differentiate between NK1 and NK2 receptors and hence between the actions of substance P and NKA [1].

The interest of the present experiments is that ir neurokinins can be released centrally from a fibre type other than nociceptors. Thus these fibres need consideration as being involved in the prolonged increases in some spinal reflexes following electrical stimulation of small diameter muscle afferents [14].

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Noxious mechanical stimulation of the hind paws of the anaesthetized rat fails to elicit release of immunoreactive β -endorphin in the periaqueductal grey matter

A.W. Duggan^a, P.J. Hope^a, C.W. Lang^a and B. Bjelke^b

^aDepartment of Preclinical Veterinary Sciences, University of Edinburgh, Edinburgh (UK) and ^bDepartment of Histology and Neurobiology, Karolinska Institutet, Stockholm (Sweden)

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As a test of the hypothesis that an animal responds to a severe peripheral painful stimulus by a central release of β -endorphin, antibody microprobes were inserted stereotactically into the midbrain of urethane anesthetized rats. These microprobes bore antibodies to β -endorphin immobilized to their outer surfaces. While microprobes were in the brain for periods of 10 to 30 min either no stimulus was delivered or alligator clamps were applied to both hind paws. Microprobes were then incubated with ¹²⁵I- β -endorphin. Quantitative image analysis of microprobe autoradiographs showed no differences between the no-stimulus and noxious-stimulus groups. Thus these experiments found no evidence for β -endorphin release following a severe peripheral painful stimulus.

Acute noxious foot shock to rats produces a naloxone reversible analgesia [14, 15] and elevated circulating levels of immunoreactive (ir) β -endorphin [10, 17]. Elevated levels of circulating β -endorphin however, do not appear to produce analgesia [17] and a central release of opioids including β -endorphin has been proposed as responsible for opioid analgesia produced by a variety of procedures. The sites where such release could occur are many including areas within the brain but also in the superficial dorsal horn of the spinal cord from activity in descending fibres [19].

The opioid β -endorphin has a relatively restricted distribution within the brain. The cell bodies synthesizing β -endorphin are found predominantly in the hypothalamic arcuate nuclei [12] and the axons arborize mainly in the hypothalamus, midbrain and medulla. Prominent among these are fibres coursing through and terminating in the periaqueductal grey matter (PAG). These PAG-terminating fibres have been implicated in mechanisms of analgesia. Thus electrical stimulation in the ventral PAG of the rat produces naloxone reversible analgesia

[1, 14]. The site of release of an opioid peptide produced by such stimulation is not known with certainty but Herz and Millan [10] have suggested that the resultant analgesia derives from a release of β -endorphin within the PAG from direct stimulation of nerve terminals adjacent to the stimulating electrodes.

One recent study [7] measured β -endorphin levels in the PAG of rats following subcutaneous injection of formalin into the forepaws and found elevated amounts at 60 and 120 minutes post injection. Elevated levels of any compound in an area of the brain when measured by homogenization and extraction probably mainly represents that present within neurones. Thus elevated levels could equally result from a phase of inhibition of release just prior to extraction or from increased synthesis following a previous period of release. Difficulties have arisen from measuring β -endorphin in cerebrospinal fluid (CSF). Early reports found that electrical stimulation in the region of the periaqueductal grey matter of humans, adequate to produce pain relief, was associated with elevated levels of β -endorphin in CSF. A subsequent study however found that the contrast media used as aids to ventricular cannulation interfered with the radioimmunoassay for β -endorphin and that increased levels could not be linked to pain relief [2]. Another procedure used for pain relief, transcutaneous vibratory

Correspondence: A.W. Duggan, Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK.

stimulation, has been recently shown not to produce changes in CSF levels of β -endorphin [8]. These difficulties associated with indirect measures of release indicate the need for a more direct approach.

Although many of the experiments cited have used electrical stimulation, and this is relevant to clinical practice, the present experiments have examined a question fundamental to an understanding of the physiology of β -endorphin: does a severe peripheral noxious stimulus evoke release of β -endorphin in the region of the periaqueductal grey?

This has been studied in anaesthetised rats by using antibody microprobes inserted through the cerebral cortex into the PAG and delivering a severe noxious mechanical stimulus to the hind paws. Although these experiments have had to be conducted with the constraint of anaesthesia, ethical considerations prevent the conduct of release experiments in conscious animals by any method when repeated noxious stimuli are used.

Experiments were performed on 6 rats anaesthetised with intraperitoneal urethane (1.5 g/kg initially and supplemented when necessary). The trachea was cannulated and blood pressure measured with a cannula in a femoral artery. Animals were mounted in a stereotaxic head frame after 2% lidocaine had been sprayed into the ears, and bilateral bone flaps were removed over the sites of proposed microprobe entry. The dura mater was removed at these sites. The exposed areas of cerebral cortex were intermittently irrigated with warm Ringer's solution. When microprobes had been introduced, small pieces of plastic film were placed over the adjacent cerebral cortex to minimize drying. A controlled electric blanket was used to maintain body temperature in the range 36–38°C. The area chosen for penetration of the PAG was A.P. +2.2 mm using the stereotaxic atlas of Paxinos and Watson.

Microprobes were prepared by immobilizing antibodies to β -endorphin to the outer surfaces of glass micropipettes [4]. Briefly the micropipettes were coated successively with: a polymer derived from gamma amino propyltriethoxysilane, glutaraldehyde, protein A and finally antibodies. In vitro tests showed that incubation of microprobes in β -endorphin, 10–8 M, for 30 minutes at 37°C resulted in near complete suppression of the binding of ^{125}I - β -endorphin (Amersham). With such sensitivity microprobes are estimated to detect 10^{-17} mol of ligand bound over 100 μm of length [3] and this is one order of magnitude better than that of microprobes used to detect release of substance P [5].

Microprobes were inserted into the brain two at a time using stepping motor microdrives. In 5 experiments the microprobes were inserted 7 mm from the brain surface at approximately the same anterior–posterior level, but

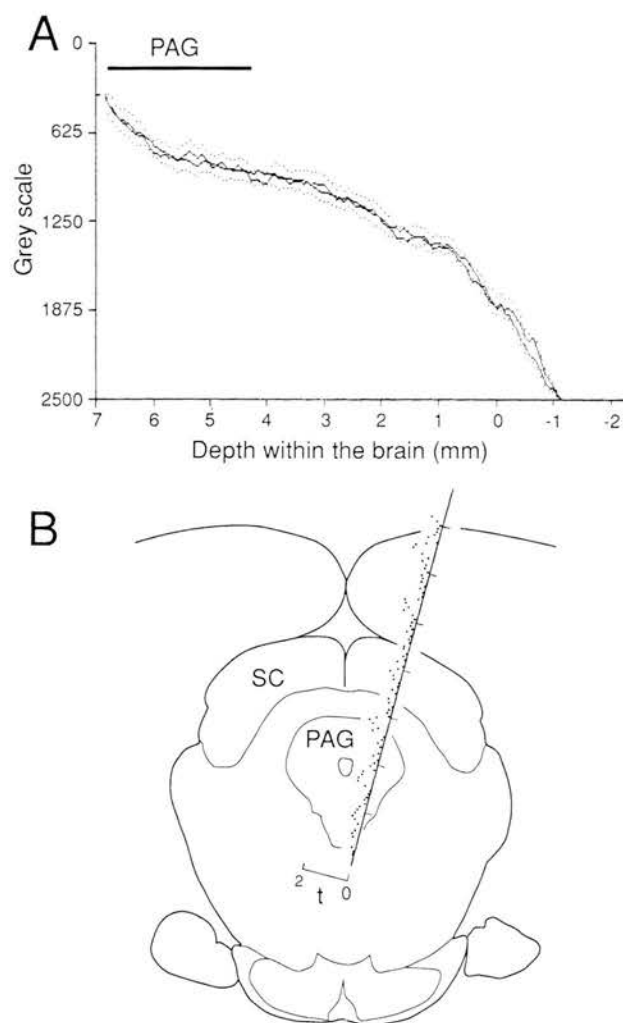


Fig. 1. A: the mean image scan of 38 microprobes inserted 7 mm into the brain along the track indicated in B, and no peripheral stimulus was applied, is plotted together with the mean scan of 31 microprobes present in the brain while a severe noxious mechanical stimulus was applied to both hind paws. The image analysis was performed at 30 μm intervals and for both groups a line has joined the mean density obtained at each point. For the no stimulus group the +S.E.M. has been plotted at each analysis point and the -S.E.M. plotted for the noxious stimulus group. Ordinate — Grey scale: integrals obtained by transverse integration of optical density of microprobe images in 30 μm intervals. Abscissa — Depth within the brain of analysis points. B: The t -statistics derived from the differences of mean density (grey scale) at each analysis point for the no stimulus and the noxious stimulus groups have been plotted along the line of introduction of microprobes into the midbrain. As the t -values are all less than 2, the observed differences are not significant at the $P < 0.05$ level.

from opposite sides, at an angle of 15° to the vertical. Such a track is illustrated in Fig. 1. In one experiment microprobes were introduced 10 mm into the brain. Microprobes remained in the brain for periods of 10 to 30 minutes, and either no peripheral stimulus was applied, or alligator clamps were applied to all of the digits of both hind paws and to the central pads with a sequence

of 3 min on, 2 min off. This stimulus was experienced as very painful by the experimenters when applied to the fingers. Approximately twenty microprobes were inserted into the brain in each experiment and animals were killed by an intravenous injection of pentobarbitone sodium.

Following removal from the brain, microprobes were first washed in cold phosphate buffered saline (PBS) and then inserted into a solution of ^{125}I - β -endorphin in PBS containing bovine serum albumin 2%. After 24 h incubation at 60°C, microprobes were again washed and the tips were then carefully broken off and glued to a sheet of paper and placed in an X-ray film cassette with a sheet of monoemulsion film (Kodak NMC). Exposures varied from 3 to 10 days.

Autoradiographs of microprobes were scanned with an image analysis system using an Imaging Technology PC Visionplus frame grabber board operating within an AT based computer [6, 9]. This performed microdensitometric estimates of microprobe images and compiled integrals derived by transverse integrations across each microprobe in 30 μm intervals. These integrals were stored on hard disc and a sorting program subsequently selected microprobes meeting designated criteria and plotted the mean integrals (\pm S.E.M.) for each group, in 30 μm intervals, and determined the significance of differences between defined groups of microprobes at each interval (see Fig. 1).

Fig. 1A illustrates the mean image analysis of 38 microprobes inserted 7 mm into the midbrain in the absence of any active peripheral stimulus and that of 31 microprobes present in the same area during noxious pinching of the skin. The two group analyses are virtually identical at all sites in the brain examined including the PAG. This is further shown in Fig. 1B which plots the statistics derived from the means of these two analyses in 30 μm intervals and these are placed along a microprobe track. This shows that at each site sampled, noxious stimulation of the hind limbs failed to produce elevated levels of ir- β -endorphin and hence failed to release this neuropeptide. In one experiment microprobes were inserted 10 mm into the midbrain and hence the areas contacted at points along such microprobes differed from those inserted 7 mm, preventing addition of these results. In this experiment the mean image analysis of 12 microprobes inserted in the absence of stimulation, showed no significant differences from that of 14 microprobes inserted while alligator clips were applied bilaterally to the hind paws.

The mechanical stimulus used in the present experiment was more severe than that which produces a release of ir substance P in the spinal cord of the anaesthetised cat [5] and rat (Hope and Lang, unpublished) and yet

failed to produce a detectable release of ir- β -endorphin in the PAG. Anaesthesia may have reduced the responses of β -endorphin releasing neurones but many naloxone sensitive events have been described in anaesthetised animals [11, 13, 18]. The results do not favour the response of an animal to a severe painful peripheral stimulus [16] being mediated in part by a release of β -endorphin within the PAG.

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Calcitonin Gene-related Peptide Causes Intraspinal Spreading of Substance P Released by Peripheral Stimulation

H.-G. Schaible¹, P. J. Hope, C. W. Lang and A. W. Duggan

Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK

¹Present address: Physiologisches Institut der Universität Würzburg, Röntgenring 9, D-8700 Würzburg, FRG

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Abstract

Experiments were performed in barbiturate-anaesthetized, spinalized cats to investigate the effect of calcitonin gene-related peptide (CGRP) on the spatial distribution of immunoreactive substance P (ir-SP) in the spinal cord released by electrical nerve stimulation and noxious mechanical stimuli. The presence of ir-SP was assessed with microprobes bearing C-terminus-directed antibodies to SP. CGRP was microinjected into the grey matter of the spinal cord near microprobe insertion sites at depths of 2500, 2000, 1500 and 1000 μm using minute amounts (in total 0.2–0.5 μl) of Ringer solution containing CGRP at a concentration of 10^{-5} or 10^{-3} M. In the untreated cord electrical stimulation of the tibial nerve (suprathreshold for all C fibres) elicited release of ir-SP which was centred in and around the lamina II. After microinjection of CGRP, stimulation-associated ir-SP was detected in a region extending from the cord surface down to the ventral horn. This pattern was similar to that observed after the microinjection of synthetic peptidase inhibitors (Duggan *et al.*, *Brain Res.*, **579**, 261–269, 1992). The large expansion of sites accessed by ir-SP was time-dependent, reaching a maximal effect within 10–40 min after microinjection of CGRP, and reversal was observed in subsequent probes. A similar expansion of the regions accessed by ir-SP after microinjection of CGRP was also observed when release of ir-SP was evoked by noxious mechanical stimulation of the toes. These results indicate that one important function of CGRP in the spinal cord may be the control of the intraspinal sites and neuronal circuits accessed by released substance P, possibly by inhibition of endopeptidases responsible for peptide degradation.

Introduction

Recently we have shown in the cat that inhibition of peptidases in the spinal cord by synthetic peptidase inhibitors causes substance P (SP), which is normally focally released in the superficial dorsal horn by electrical nerve stimulation and noxious mechanical stimulation of the foot, to spread widely away from sites of release (Duggan *et al.* 1992). This was demonstrated by using antibody-bearing microprobes which allow a spatial analysis of the distribution of a neuropeptide within the spinal cord (Duggan *et al.*, 1988a; Hendry *et al.*, 1988). In the untreated spinal cord the release of immunoreactive SP (ir-SP) evoked by electrical nerve stimulation is mainly centred in and around the substantia gelatinosa, which is the major projection site of afferent fibres containing SP (Duggan *et al.*, 1988b). After the microinjection of synthetic endopeptidase inhibitors, ir-SP was detected in an area which extended from the surface of the cord down to the ventral horn, at the time of the maximal peptidase inhibitor effect (Duggan *et al.*, 1992).

These results imply that the access of tachykinin receptor sites by the ligand SP may be controlled dynamically by the activity level of endopeptidases. They support experimentally the proposal that peptides, after release, may diffuse to reach receptors in remote areas (Agnati *et al.*, 1986; Fuxe and Agnati, 1991).

Although protecting a synaptically released compound from degradation is usually considered in terms of enhancing and prolonging the event occurring at the releasing synapse, our experiments with peptidase inhibitors suggest that access to remote areas is an important physiological consequence of inhibition of degradation for compounds released in the central nervous system. Whereas the synthetic peptidase inhibitors allowed the demonstration of spreading following synaptic release in principle, a more important task is to identify endogenous compounds which influence the effect of endopeptidases and thus could act as determinants of the sites accessed by a neuropeptide. It was

Correspondence to: Professor A. W. Duggan, as above

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suggested by Le Greves *et al.* (1985) that calcitonin gene-related peptide (CGRP) may act as a physiological peptidase inhibitor. CGRP is contained in a proportion of somatic and visceral afferent fibres, with significant CGRP immunoreactivity in laminae I, II and V of the spinal cord, and a significant depletion of ir-CGRP in these laminae occurs after dorsal rhizotomy (Rosenfeld *et al.*, 1983; Gibson *et al.*, 1984; Wiesenfeld-Hallin *et al.*, 1984; Lee *et al.*, 1985; Skofitsch and Jacobowitz, 1985; Franco-Cereceda *et al.*, 1987; Ju *et al.*, 1987; Cameron *et al.*, 1988; Carlton *et al.*, 1988; Chung *et al.*, 1988; Harmann *et al.*, 1988; McNeill *et al.*, 1988; Mulderry *et al.*, 1988; Kruger *et al.*, 1989; Sharkey *et al.*, 1989; Nahin *et al.*, 1991). CGRP is often colocalized with SP in unmyelinated afferent neurons (Gibson *et al.*, 1984; Wiesenfeld-Hallin *et al.*, 1984; Gibbins *et al.*, 1985; Lee *et al.*, 1985; Matsuyama *et al.*, 1986). CGRP has been shown to cause a small depolarization of spinal cord neurons (Miletic and Tan, 1988; Ryu *et al.*, 1988). When applied topically to the spinal cord, however, CGRP has prolonged the facilitation of a flexor reflex of the rat by SP (Wiesenfeld-Hallin *et al.*, 1984; Woolf and Wiesenfeld-Hallin, 1986). One possible mechanism of interaction could be by prolonging the life of substance P by control of endopeptidase activity. On the basis of our recent findings with the synthetic peptidase inhibitors we examined in the present experiments whether microinjection of CGRP into the spinal cord would mimic the effect of synthetic peptidase inhibitors, i.e. lead to an expansion of the intraspinal area which is accessed by SP focally released in the superficial dorsal horn. The occurrence of such an action has important implications for the receptors accessed by, and hence the function of, SP in the spinal cord.

Materials and methods

Microprobe preparation

Conventional glass micropipettes were used to prepare microprobes as recently described (Duggan *et al.*, 1988a). Micropipettes, heat-sealed at both ends, were incubated in a 10% solution of aminopropyltriethoxysilane in toluene. Subsequent heat curing produced a siloxane polymer on the outer surface. Using glutaraldehyde, protein A was immobilized to the siloxane polymer in order to bind antibodies to SP during incubation of the micropipettes in a SP antiserum. We used a polyclonal antiserum which was directed against the C-terminus. The manufacturer (Peninsula) indicated for this antibody significant cross-reactivity with SP 3-11, little for SP 4-11 and no cross-reactivity for neurokinin A, neurokinin B and neuropeptide K. In *in vitro* tests (Bolton-Hunter) [125 I]SP was bound to the probes and ~50% suppression of the binding of radioactive SP was achieved by preincubation in a 10^{-7} M SP solution for 30 min at 37°C. The binding was not suppressed by preincubation in Ringer solution containing CGRP up to a concentration of 10^{-3} M.

Animal preparation

Anaesthetized, spinalized cats were used to perform the experiments. The anaesthesia was started by 35 mg/kg sodium pentobarbitone i.p. and, after cannulation of a cephalic vein, maintained by continuous infusion at 3.0 mg/kg/h. Blood pressure was measured using a cannula in a carotid artery. A tracheotomy was made for artificial respiration, which was started after the dissection following neuromuscular paralysis with gallamine, 4 mg/kg/h. End-tidal CO₂ levels and body temperature were monitored throughout the experiment. The animals were kept at 36–38°C by a controlled heating pad.

A laminectomy exposed the caudal lumbar segments. The cord was

transected at the thoracolumbar junction following injection of 0.1 ml of 2% lignocaine. The cats were fixed in a metal frame. After cutting the dura a layer of agar in Ringer was placed over the dorsal surface of the spinal cord. At the insertion sites of the probes the agar was removed and the pia-arachnoid was opened with sterile fine forceps. These exposed areas were continuously irrigated with sterile Ringer solution maintained at 38°C by a heat exchanger around the tubing interposed between the infusion pump and the spinal cord. For electrical stimulation the tibial nerve of the left hindlimb was dissected after incision of the skin and separating the two gastrocnemius muscles. A small pool was formed with the skin flaps and filled with mineral oil, and a stimulation electrode was placed under the nerve for *en passant* stimulation.

Experimental protocol

Sites were selected for the study of release of SP which showed, in extracellular recordings, strong afferent input from the electrical stimulation of the tibial nerve and from pinching the ipsilateral paw. Pairs of microprobes were introduced to a depth of 4 mm using two stepping motor micromanipulators and kept in the cord for 10 min. During these periods either no stimulus was applied, or electrical or noxious mechanical stimulation was performed. With nerve stimulation, electrical pulses of 0.5 ms duration and 50 V amplitude were applied with a frequency of 20 Hz either for 10 min (in initial experiments) or for 2 min. Since the stimulus threshold for the largest fibres was 100–200 mV, this stimulation was considered supramaximal for all afferent fibres. For noxious mechanical stimulation, five crocodile clips were applied to the toes for 3 min, and this was repeated once within the 10 min during which the microprobes were in the spinal cord. First the left side of the cord and electrical nerve stimulation were used, and then we switched to the right side of the cord and noxious mechanical stimulation was employed.

After having inserted several probes in the control period, in periods of no stimulation, electrical nerve stimulation or noxious mechanical stimulation of the toes, CGRP was microinjected into the grey matter using a micropipette connected to a syringe. Minute amounts (in total 0.2–0.5 μ l) of Ringer solution containing CGRP at 10^{-5} or 10^{-3} M were injected at depths of 2500, 2000, 1500 and 1000 μ m, around the region where the microprobes were introduced. In some experiments only Ringer solution was microinjected. Following the microinjections further probes were inserted at defined intervals after injection (starting 5 min after the microinjections were finished) and the electrical or mechanical stimuli were repeated.

At the end of the experiments pontamine sky blue was ejected at depths of 1 and 4 mm from the dorsal surface of the cord. The cords were removed, processed and cut for determination of the location of the resultant dye spots. These data were used to relate the position of the probes to the laminae of the grey matter.

Treatment of probes and data analysis

After removal from the spinal cord, the microprobes were washed for 15 min in cold phosphate-buffered saline (PBS) containing Tween (0.1%) and then incubated for 24 h at 6°C in a PBS–azide solution of [125 I]SP containing bovine serum albumin. The final dilution of the radiolabelled peptide was ~2000 dpm per μ l. Following incubation in labelled peptide the probes were washed for 15 min in Tween, and the tips were broken off and placed in an X-ray cassette on a monoemulsion film (Kodak NMC) for up to 7 days.

The resulting X-ray images were scanned at 10- μ m intervals using

a CCD camera and an image analysis system employing an Imaging Technology PC Vision Plus frame grabber board operating in a DCS 286e (AT-based) computer. This procedure produced an image of each probe showing the grey density along 5 mm of the tip. Transverse integrations were then performed along each analysed image to give a plot of the total optical density (grey scale) with respect to length. Although the image analysis system produced a plot with a longitudinal resolution of 10 μm , this was reduced to 30 μm by calculating the average of three successive integrals. This is more in line with the biological resolution of the microprobe method (Duggan, 1991). Inhibition of binding of radioactive SP on these probes (reduced grey density) indicates previous binding of ir-SP in the cord. These sites of deficits were then related to sites within the cord (see above). Groups of probes were pooled and the means \pm SEM were displayed in graphs which showed the averaged grey densities along the probes in relation to the depth within the cord. Comparisons were made between different groups of probes using the Student's *t*-test at intervals of 30 μm .

Results

Pattern of the intraspinal release of immunoreactive substance P evoked by electrical nerve stimulation

Control conditions

In each experiment the first pair of probes was inserted into the spinal cord prior to electrical nerve stimulation ('No stim.' probes), and the subsequent two pairs of probes were introduced whilst the tibial nerve was stimulated electrically ('Nerve stim. control' probes). All No stim. and Nerve stim. probes were kept in the spinal cord for periods of 10 min and the intervals between the insertion of subsequent pairs of probes were 10–15 min.

Prior to electrical stimulation there was no substantial release of ir-SP. By contrast, significant release of ir-SP in the dorsal horn was evoked by electrical stimulation of the tibial nerve in all cats. Figure 1A shows the averaged images of the No stim. probes ($n = 19$) and of Nerve stim. control probes ($n = 23$). The image of the No stim. probes shows a small zone of reduced binding of [^{125}I]SP at ~ 1 mm from the dorsal surface which did not, however, differ significantly from that of probes incubated in a solution of [^{125}I]SP without previous exposure to the non-labelled neuropeptide. During the presence of the Nerve stim. control probes in the cord the tibial nerve was stimulated electrically for 2 min with pulses of 0.5 ms duration and an amplitude of 50 V delivered at a frequency of 20 Hz. These probes show an inhibition of binding [^{125}I]SP from ~ 0.5 mm to ~ 2.0 mm from the dorsal surface with a peak at 1 mm. This deficit of radioactive binding is equated with previous binding to the probe of SP which was released in the cord by nerve stimulation. The statistical analysis of the differences between the No stim. and the Nerve stim. control curves is displayed in Figure 2A (see below).

Microinjection of CGRP

After having examined the release of ir-SP under control conditions, CGRP (10^{-5} M solution) was microinjected into the grey matter around the insertion sites of the probes at depths of 2500, 2000, 1500 and 1000 μm (four tracks). The total volume injected was 0.5 μl and the injection procedure took ~ 10 min. At defined intervals after the last microinjection, further pairs of probes were inserted and the tibial nerve was stimulated electrically with the same parameters as in the control period (for 2 min with pulses of 0.5 ms duration and an amplitude of 50 V delivered at a frequency of 20 Hz). Again the

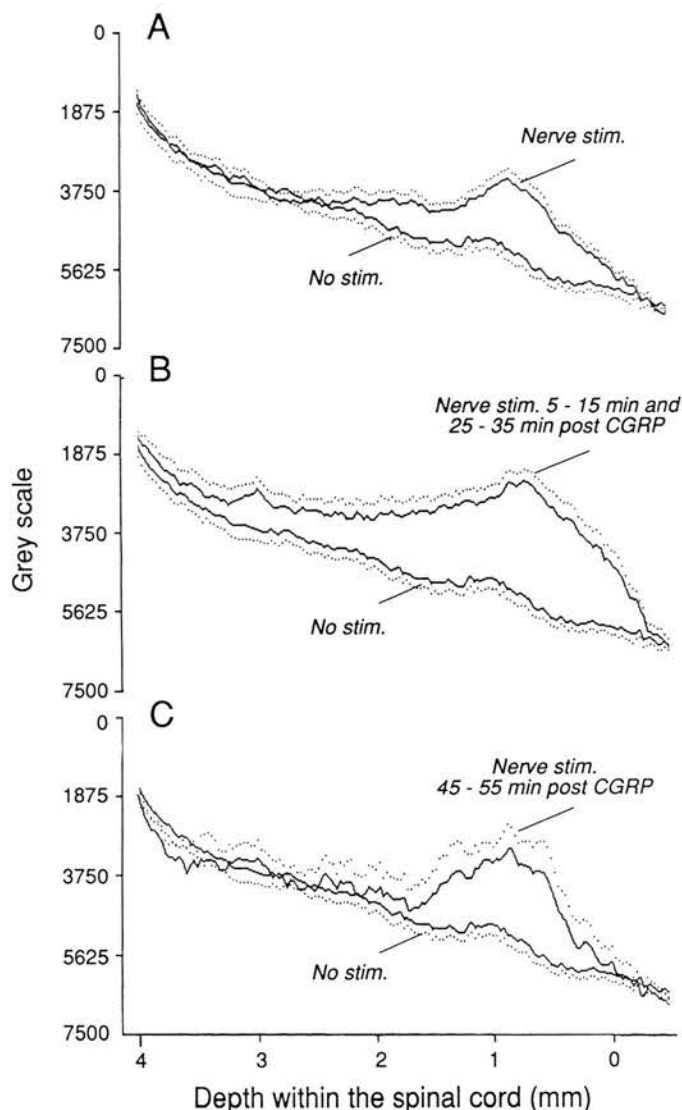


FIG. 1. Pattern of the stimulus-evoked release of ir-SP in the untreated cord (A) and after CGRP (10^{-5} M) microinjected at depths of 2500, 2000, 1500 and 1000 μm (B and C). Each curve represents the averaged densitometric plot of a sample of probes which have bound [^{125}I]SP and produced autoradiographic images. The lines are the connected mean values at 30- μm intervals and the dots represent the standard errors at these intervals. Prior to incubation in [^{125}I]SP the probes were inserted 4 mm into the cord for periods of 10 min either prior to nerve stimulation ('No stim.', $n = 19$) or during electrical stimulation of the tibial nerve ('Nerve stim.' probes; stimulus duration 2 min, impulses of 50 V, 0.5 ms duration, delivered at a frequency of 20 Hz). In all graphs 0 mm corresponds to the surface of the cord, 4 mm corresponds approximately to the ventral border of the ventral horn, and 1 mm corresponds approximately to lamina II. The slope of the No stim. probes reflects the shape of the microprobes with an increasing diameter, and hence the number of binding sites along the shaft. Inhibition of binding of [^{125}I]SP (reduced grey density) is equated with previous binding of (unlabelled) endogenous ir-SP in the cord.

intervals between the introduction of pairs of probes were ~ 10 –15 min.

When CGRP was previously microinjected into the grey matter, the microprobes present in the spinal cord during nerve stimulation showed a different pattern of [^{125}I]SP binding. Figure 1B shows the combined

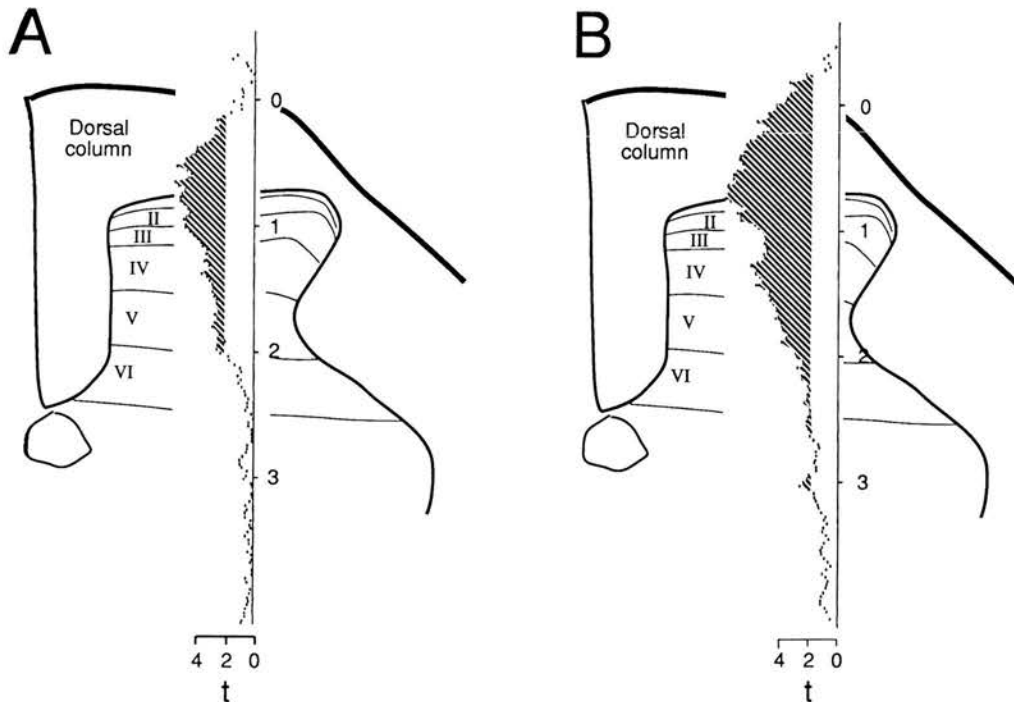


FIG. 2. Statistical analysis of the data shown in Figure 1 superimposed on schematic drawings of transverse sections of the spinal cord. The graphs show the significance of the differences between the averaged images of the No stim. probes and the Nerve stim. probes (in A) and the significances of the differences between the images of the No stim. probes and the 'Nerve stim. 5–15 and 25–35 min post CGRP' probes (in B). The significances were calculated at 30 μ m intervals and plotted along the probe. 2t corresponds to $P < 0.05$ and 4t to $P < 0.01$. The shaded areas indicate sites in the cord in which the stimulus curves were significantly different from the non-stimulation baseline, with $P < 0.05$. The numerals correspond to the laminae of Rexed.

averaged images of two groups of probes. They were in the cord either 5–15 or 25–35 min after microinjection of CGRP. Compared to the No stim. image the grey density on these probes ($n = 12$) was reduced over a zone extending from the dorsal surface to ~ 3.3 mm ventrally. This pattern indicates previous binding of endogenous ir-SP to the probes in a large area of the cord during peripheral nerve stimulation. Probes in the cord 45–55 min after microinjection of CGRP ($n = 5$) showed a considerable reversal of the effect of CGRP. The image of the 'Nerve stim. 45–55 min post CGRP' probes (Fig. 1C) shows a partial return to the image of the Nerve stim. probes displayed in Figure 1A. There was a difference from the No stim. baseline only in an area from 0.3 to 1.7 mm.

Figure 2 illustrates the changes in the area over which ir-SP was detected following microinjection of CGRP in relation to spinal laminae. Figure 2A is derived from the probes shown in Figure 1A (electrical stimulation prior to CGRP) and Figure 2B was compiled from the probes displayed in Figure 1B (effect of CGRP on stimulus-evoked ir-SP.) In both cases the statistics derived from the differences between No stim. probes and Nerve stim. probes are plotted at 30- μ m intervals. The shaded areas in both graphs show the areas in which significance of the differences was at least $P < 0.05$ (2t). In the untreated cord the area of significant stimulus-evoked ir-SP release was extended from the white matter adjacent to the superficial dorsal horn to lamina V with a peak in the region of lamina II (Fig. 2A). After microinjection of CGRP the area of significant stimulus-associated ir-SP reached the dorsal surface of the cord and even lamina VII (Fig. 2B).

Microinjection of CGRP versus Ringer solution

In further experiments the effect of microinjection of Ringer solution was compared to that of microinjection of CGRP. After having tested

the release of ir-SP in the control period, Ringer solution was microinjected using the same protocol as with CGRP and then pairs of probes were inserted and the tibial nerve was stimulated electrically. Finally, CGRP (10^{-3} M) was microinjected at the same site and the stimulation was repeated using further pairs of probes for the detection of ir-SP. In all of these experiments the tibial nerve was stimulated with pulses of 50 V, 0.5 ms at a frequency of 20 Hz for periods of 10 min.

Whereas the microinjection of CGRP caused an expansion of the area in which stimulus-evoked ir-SP was detected, the microinjection of Ringer did not produce this effect. This is displayed in Figure 3A. It shows the averaged images of No stim. probes ($n = 19$, the same groups as in Fig. 1), 'Nerve stim. post Ringer' probes ($n = 14$) and 'Nerve stim. 25–35 min post CGRP' probes ($n = 13$), all of which were present in the cord for periods of 10 min. After the microinjection of Ringer the pattern of stimulus-evoked ir-SP was similar (and statistically not different) to that found when the cord was left untreated prior to stimulation. The effect of CGRP was similar to that obtained with a solution of 10^{-5} M (see Fig. 1). Figure 3B shows the statistical differences between the Nerve stim. post Ringer and the Nerve stim. post CGRP probes. Although levels of ir-SP were increased at the site of maximal release (1 mm from the dorsal surface) following CGRP, the greatest and most significant changes occurred both dorsally and ventrally to this area.

Microinjection of CGRP versus peptidase inhibitors

In a previous study we examined the effect of microinjection of the peptidase inhibitors kelatorphan and enalaprilat (inhibitors of endopeptidase 3.4.24.11 and angiotensin-converting enzyme respectively) on the pattern of ir-SP evoked by electrical stimulation

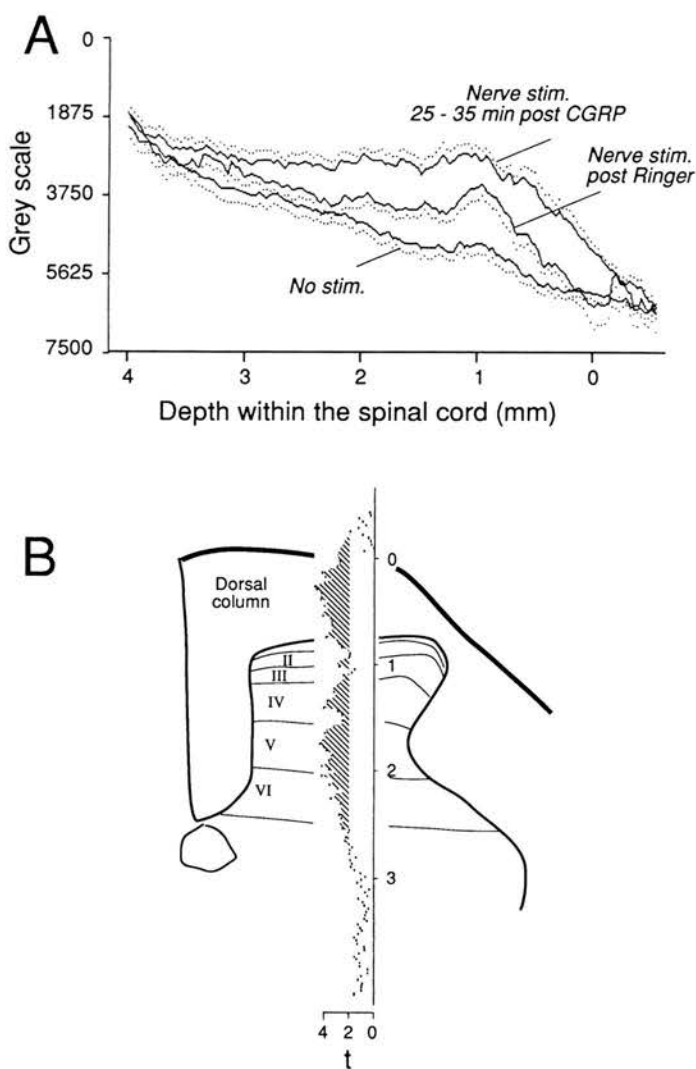


FIG. 3. Effect of microinjection of Ringer and of CGRP (10^{-3} M) on the pattern of stimulus-evoked ir-SP. (A) Averaged images of the 'No stim.' probes ($n = 19$), 'Nerve stim. post Ringer' probes ($n = 14$) and 'Nerve stim. 25–35 min post CGRP' probes ($n = 13$). Electrical stimulation of the tibial nerve for 10 min with pulses of 50 V, 0.5 ms duration, at a frequency of 20 Hz. (B) Statistical analysis between the Nerve stim. 25–35 min post CGRP probes and the Nerve stim. post Ringer probe shown in A. The same analysis was used as in Figure 2.

of the tibial nerve (Duggan *et al.*, 1992). Since the experimental protocol in the previous and the present study was similar, the images of microprobes sampled during comparable resting and stimulus conditions can be related to each other. For this comparison the groups of probes with the maximum effects of the compounds were chosen, i.e. for peptidase inhibitors (10^{-3} M) the probes present in the cord 35–45 min after microinjection, and for CGRP (10^{-3} M) the probes inserted in the cord 25–35 min after application of CGRP. During exposure of all probes the tibial nerve was stimulated electrically for 10 min. Figure 4 shows that the average image of probes present in the cord after peptidase and after CGRP were very similar. Except for a small area in the dorsal columns there was no statistically significant difference between these groups of probes. This result

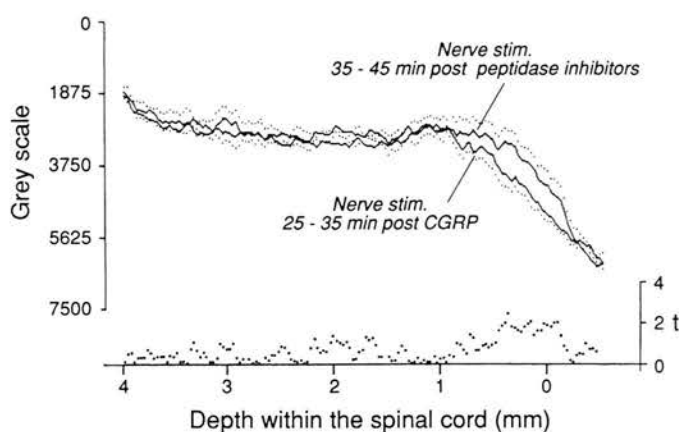


FIG. 4. Effect of microinjection of CGRP and of the peptidase inhibitors kelatorphan and enalaprilat on the stimulus-evoked release of ir-SP. The release of ir-SP was evoked by electrical stimulation of the tibial nerve for 10 min with pulses of 50 V, 0.5 ms pulse duration, at 20 Hz. The plots represent the averaged images of probes present in the cord under the conditions indicated. For both compounds the subgroup of probes was selected which showed the maximal effect after the injection of the compound(s). For the 'Nerve stim. post CGRP' group the number of probes was 13 and for the 'Nerve stim. post peptidase inhibitors' group it was 15. The dots over the x-axis show the statistical analysis.

suggests that the effects of CGRP and synthetic peptidase inhibitors on the pattern of stimulus-evoked ir-SP were similar, i.e. possibly involving similar mechanisms.

Pattern of the intraspinal release of ir-SP evoked by noxious mechanical stimulation

In another series of probes we examined whether CGRP also had an effect on the release of ir-SP evoked by noxious pinch applied to restricted areas, thus activating a smaller number of afferents than electrical nerve stimulation. After having studied in the left side of the lumbar cord the release of ir-SP evoked by electrical stimulation of the tibial nerve, further probes were inserted into the right side of the cord and noxious pinch was used as a stimulus. Five crocodile clips were applied to the ipsilateral toes and the foot pad, twice for 3 min in a 10-min test period. The experimental protocol was similar to that used with electrical nerve stimulation.

Figure 5 shows the result of this series of experiments. In all graphs the averaged image of Nox. pinch probes is related to the averaged image of No stim. probes, i.e. the image of 21 probes which were in the cord prior to any noxious mechanical stimulation. Figure 5A shows the release of ir-SP during pinch whilst the cord was left untreated ($n = 46$ probes). There was a small zone of release at about 1 mm. Figure 5B, C shows the release of ir-SP following the microinjection of CGRP (10^{-3} M) at depths of 2500, 2000, 1500 and 1000 μ m (total volume ~ 0.5 μ l). Again there was an increase in the difference between the Nox. pinch and the No stim. curves, and the zone of stimulus-evoked ir-SP showed an expansion which developed with time after the injection.

Discussion

These results show that the presence of CGRP in the spinal cord may alter the pattern of distribution of ir-SP evoked by electrical nerve and noxious mechanical stimulation. Whilst the release of ir-SP is usually

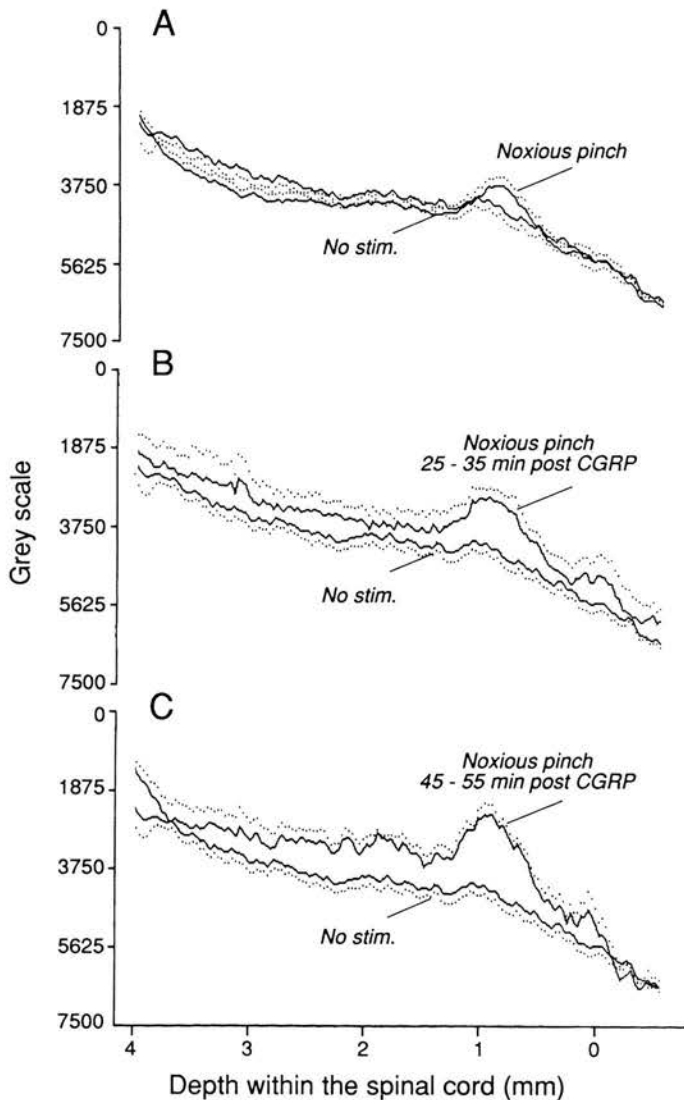


FIG. 5. Effect of microinjection of CGRP at 2500, 2000, 1500 and 1000 μm on the pattern of release of ir-SP evoked by noxious mechanical stimulation. Each graph shows the averaged image of the 'No stim.' probes of the control period ($n = 21$) and the averaged image of probes present in the cord during noxious pinch under the conditions indicated ($n = 46$ in A, 7 in B, and 6 in C). Each probe was in the cord for 10 min; noxious pinch of the toes was applied twice for 3 min in the 10-min periods.

more focal and centred in and around lamina II, this neuropeptide can be detected in a large area of the cord after microinjection of CGRP. It was essential to show that the decreased binding of [^{125}I]SP to microprobes was not due to binding by the microinjected CGRP. *In vitro* tests indicated that CGRP did not suppress the binding of [^{125}I]SP to the probes. It was also important to examine whether the microinjection itself could alter the pattern of binding of ir-SP. Probes which were present in the cord during electrical nerve stimulation, after microinjection of Ringer solution, showed a pattern which almost overlapped that found on probes present in the untreated cord during nerve stimulation. Collectively, therefore, the pattern of decreased binding of [^{125}I]SP after CGRP most likely resulted from the widespread presence of ir-SP in the dorsal horn as a consequence of

some action of CGRP. This result indicates, therefore, (1) that the presence of ir-SP in a defined area of the cord is dependent on factors additional to the site of release, and (2) that CGRP is an endogenous compound with the capacity to alter the sites accessed by ir-SP.

Increased levels of ir-SP in the spinal cord following the administration of CGRP could result either from an enhanced release mechanism or by an inhibition of the degradation of SP. Oku *et al.* (1987) proposed an enhancement of release when they found that CGRP increased the levels of ir-SP released into the perfusate of a rat spinal cord slice preparation by capsaicin, although CGRP alone did not alter the basal (unstimulated) release of ir-SP. As mentioned above, under *in vitro* conditions CGRP inhibits an endopeptidase degrading SP (LeGreves *et al.*, 1985). If degradation is prevented, ir-SP could achieve higher concentrations at site(s) of release and hence could diffuse to remote areas. We favour the second mechanism since in our previous study the pattern of ir-SP was altered in the same way after CGRP as by the microinjection of synthetic peptidase inhibitors.

The effect of CGRP on the spatial distribution of ir-SP could be demonstrated using either electrical nerve stimulation or application of noxious pinch. The development and the size of the effect of CGRP differed to some extent with the two types of stimulation. With electrical nerve stimulation the effect of CGRP was much more pronounced, probably as a result of the activation of a much larger number of afferent fibres. Furthermore, the expansion of ir-SP developed rapidly and showed reversibility during the time period studied (Fig. 1). With noxious pinch the expansion of released ir-SP seemed to be slower. The reasons for these differences in the time course are not known but the findings suggest that several factors, such as the amount of released ir-SP and the local presence of CGRP, may interact.

CGRP is released from slices of the spinal cord and cultured dorsal root ganglion cells *in vitro* by solutions with a high extracellular potassium concentration and/or capsaicin (Mason *et al.*, 1984; Saria *et al.*, 1986; Pohl *et al.*, 1989). Intraspinal release *in vivo* has been shown with application of electrical stimulation of unmyelinated afferent fibres and noxious mechanical stimuli (Morton and Hutchison, 1989). Co-release of CGRP and SP was demonstrated from the peripheral branches of capsaicin-sensitive afferents (Geppetti *et al.*, 1989; Maggi *et al.*, 1991) as well as from spinal cord slices (Saria *et al.*, 1986). It is very likely, therefore, that many stimuli, especially noxious ones, lead to co-release of ir-CGRP and ir-SP from the central terminals of afferent neurons. The normal stimulus-evoked release of ir-SP in our experiments may therefore represent a process of release and partial persistence of ir-SP resulting from peptidase inhibition by co-released CGRP. This effect could explain why the zone of stimulus-evoked release in the control conditions was apparently larger than the region in which SP-positive neurons are mainly found.

The functional significance of the present results may be related to the spatial distribution of receptors for SP. Although the location of NK1 receptors in the spinal cord matches in general the location of SP-positive neurons and fibres, the density of receptors and SP-immunoreactive neurons may differ (Ljungdahl *et al.*, 1978; Gibson *et al.*, 1984; Charlton and Helke, 1985; Lee *et al.*, 1985; Helke *et al.*, 1986; Yashpal *et al.*, 1990). Some of the receptors are located in regions remote from the areas in which the SP-containing neurons are located preferentially and in which ir-SP is released under normal conditions. If SP diffuses after stimulation it could reach and activate these binding sites. The presence of other factors, including the release of CGRP, therefore, may determine when receptors for SP will be activated.

Previous experiments with antibody microprobes showed that the normal release pattern for neurokinin A (NKA) in the spinal cord

resembled that of SP following CGRP in the present experiments (Duggan *et al.*, 1990; Hope *et al.*, 1990). It was proposed that this resulted from a slow degradation of synaptically released NKA. *In vitro* experiments have shown a rapid degradation of SP but not NKA by plasma (Theodorsson-Norheim *et al.*, 1987), angiotensin-converting enzyme (Hooper *et al.*, 1985) and an endopeptidase isolated from human cerebrospinal fluid (Nyberg *et al.*, 1984). With the isolated rat urinary bladder the proposed action of NKA relative to that of SP has been attributed to a slow degradation of NKA by peptidases (Maggi *et al.*, 1991). It is a fascinating possibility that the central terminals of nociceptors release a focally acting compound (such as L-glutamate), a relatively non-focal compound (SP) and a diffusely acting substance (NKA), but that the degree of diffuseness of the action of SP is under the control of another co-released peptide, CGRP. This concept of non-focal action of synaptically released compounds has recently been summarized by Fuxe and his colleagues and termed volume transmission (Agnati *et al.*, 1986; Fuxe and Agnati, 1991).

A synergistic action of SP and CGRP at the spinal level has been described in some situations. These peptides act synergistically on the gain of the flexor withdrawal reflex in the rat which is elicited by stimulation of unmyelinated primary afferent fibres (Woolf and Wiesenfeld-Hallin, 1986). Ionophoretic application of both SP and CGRP onto dorsal horn neurons in the spinal cord of the rat showed that CGRP enhanced the excitatory effect of SP, but did not elevate resting discharges on its own; it also enhanced the SP-mediated facilitation of responses to cutaneous noxious stimuli (Biella *et al.*, 1991). In behavioural experiments the intrathecal application of SP and CGRP together has a longer effect on the mechanical pain threshold than SP alone, although the amount of facilitation by SP is not changed by CGRP (Oku *et al.*, 1987). Such effects may result from postsynaptic receptor-mediated action of both neuropeptides, but they are also consistent with a prolonged life of SP through endopeptidase inhibition by CGRP.

Acknowledgements

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Abbreviations

CGRP	calcitonin gene-related peptide
ir	immunoreactive
NKA	neurokinin A
PBS	phosphate-buffered saline
SP	substance P

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Analgesic doses of morphine do not reduce noxious stimulus-evoked release of immunoreactive neurokinins in the dorsal horn of the spinal cat

C.W. Lang,¹ A.W. Duggan & P.J. Hope

Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH

- 1 Antibody microprobes were used to detect immunoreactive neurokinin A release in the dorsal spinal cord of barbiturate-anaesthetized spinal cats.
- 2 Noxious mechanical stimulation of the ipsilateral hind paw and electrical stimulation (suprathreshold for unmyelinated primary afferent fibres) of the ipsilateral tibial nerve evoked immunoreactive neurokinin A release.
- 3 Systemic morphine, 5 mg kg⁻¹, i.v., did not block immunoreactive neurokinin A release in response to these stimuli.
- 4 Subsequent naloxone administration, 0.5 mg kg⁻¹, i.v., did not alter this stimulus-evoked release.
- 5 Basal levels of immunoreactive neurokinin A were unaltered by morphine or naloxone.
- 6 These results suggest that the analgesic effects of morphine at the spinal cord level are not brought about by activation of presynaptic opiate receptors on neurokinin A containing afferent terminals.

Keywords: Neurokinins; morphine; naloxone; antibody microprobes; cat spinal cord.

Introduction

Substance P and neurokinin A, two members of the tachykinin family of neuropeptides, extensively coexist in the small diameter dorsal root ganglion cells of the rat (Dalsgaard *et al.*, 1985). Both tachykinins have been proposed as important in the transmission of information from nociceptive afferents to spinal or brainstem neurones (Otsuka & Yanagisawa, 1988; Fleetwood-Walker *et al.*, 1990). Studies of the release of these compounds in the spinal cord by use of antibody microprobes have shown both tachykinins to be released in response to a similar range of peripheral noxious stimuli (Duggan *et al.*, 1988b; 1990; Hope *et al.*, 1990a; Schaible *et al.*, 1990) although important differences in noxious stimuli release patterns have also been identified. Noxious stimuli producing detectable tissue damage caused greater release of immunoreactive substance P in the dorsal horn, than noxious, non-damaging stimuli. Noxious cutaneous stimuli also released immunoreactive neurokinin A, but potentiation by tissue damage was not seen. Immunoreactive neurokinin A exhibited a remarkable spread beyond sites of release and persisted following an effective stimulus. In contrast a focal, non-persistent pattern was observed with immunoreactive substance P. These findings suggest that neurokinin A, unlike the relatively short-lived substance P, may not function as a conventional neurotransmitter, but might rather have a long term modulatory role on spinal cord systems.

Iontophoretic administration of morphine (Duggan *et al.*, 1977) and the μ opioid agonist [D-Ala² Me-Phe⁴, Gly-ol⁵] enkephalin (DAMGO) (Fleetwood-Walker *et al.*, 1988) in the region of the substantia gelatinosa selectively suppressed the transmission of impulses from nociceptive afferents to deeper neurones of the dorsal horn. The substantia gelatinosa is the major site of termination of unmyelinated cutaneous afferent fibres (Light & Perl, 1977). Since administration of naloxone in the substantia gelatinosa completely blocked the effects of analgesic doses of systemic morphine on deep neurones of the dorsal horn of the cat (Johnson & Duggan, 1981) it is likely that opiate receptors in the substantia gelatinosa are the major ones mediating the analgesic actions of morphine at the spinal level, whether administered systemically or intrathecally. One mechanism by which morphine could produce

analgesia is by presynaptic inhibition of neurotransmitter release from nociceptive primary afferent terminals. In support of this hypothesis, morphine in high concentrations, when added to the superfusate of either a slice of the upper dorsal horn (Jessel & Iversen, 1977; Mauborgne *et al.*, 1987) or of the spinal cord *in vivo* (Yaksh *et al.*, 1980; Go & Yaksh, 1987) has been shown to reduce stimulus evoked release of immunoreactive substance P. However, systemically administered morphine at analgesic doses has failed to reduce release of immunoreactive substance P evoked by noxious stimulation (Kuraishi *et al.*, 1983; Morton *et al.*, 1990).

With the recognition that a peripheral noxious stimulus results in the central release of a number of neuroactive compounds including amino acids (Kangrga & Randic, 1990) and peptides (Duggan *et al.*, 1988b; 1990), a complete study of opiates and release from primary afferents is clearly a complex task. There is evidence that the arrival of impulses in nociceptive afferents produces not only fast transmission to fibres ascending to the brain but also long lasting events such as prolonged facilitation of flexor reflexes (Woolf, 1983; Woolf & Wall, 1986a; Cook *et al.*, 1987; Cervero *et al.*, 1988; Neugebauer & Schaible, 1988; Hoheisel & Mense, 1989; Hylden *et al.*, 1989). It has been proposed that neurokinin A (rather than substance P) is more important in these prolonged spinal events since, following release of immunoreactive neurokinin A, this peptide (or an extended form such as neuropeptide K) diffuses widely and persists in the spinal cord (Hope *et al.*, 1990a). Of note is the finding that in the rat, low doses of morphine suppressed the prolonged facilitation of spinal reflexes produced by nociceptive input, whereas higher doses were required to suppress fast transmission as measured by an unfacilitated nociceptive reflex (Woolf & Wall, 1986b). Such considerations indicate the need for a study of opiates and the central release of neurokinin A. In the present study therefore, the effects of analgesic doses of systemically administered morphine were examined on the noxious stimulus-evoked release of immunoreactive neurokinin A in the dorsal horn of the spinal cord in barbiturate-anaesthetized cats.

Methods

Preparation of antibody microprobes

Antibody microprobes were prepared as previously described (Duggan *et al.*, 1988a). Fine glass micropipettes, heat sealed at

¹ Author for correspondence.

base and tip, were incubated in a 10% solution of amino-propyltriethoxysilane producing an even layer of a siloxane polymer bearing free amino-groups on the outer surface of the probes. Glutaraldehyde was then used to immobilize protein A (Sigma) onto this polymer; protein A then bound immunoglobulins present in an antiserum raised against the carboxyl terminus of neurokinin A (Peninsula). Although this antiserum is described as anti-neurokinin A, information from the manufacturer indicates it has 100% cross reactivity with neuropeptide K (an N-terminal extended form of neurokinin A), 70–80% cross-reactivity with neurokinin B, but negligible cross-reactivity with substance P. For purposes of the present experiments microprobes are described as detecting immunoreactive neurokinins.

The antiserum lyophilate contained salts from the suspending buffer which limited the possible concentration of resuspended antibody. A dilution of approximately 1:3000 was used. All protein binding outside the spinal cord took place in 5 μ l glass capillaries containing the relevant solutions.

Animal preparation/recording/microprobe analysis

Experiments were performed on 8 cats anaesthetized initially with intraperitoneal sodium pentobarbitone 35 mg kg⁻¹, and maintained by continuous intravenous infusion of sodium pentobarbitone, 3 mg kg⁻¹ h⁻¹. All animals were artificially ventilated with air and when necessary paralysed with gallamine triethiodide, 4 mg kg⁻¹ h⁻¹. Blood pressure was measured continuously via a cannula in the left carotid artery and end tidal CO₂ levels were also monitored and maintained at 4%.

The lumbar spinal cord was exposed by dorsal laminectomy and the spinal cord transected at the level of T13/L1 following injection of 0.1 ml of 2% lignocaine (Astra). The lumbar dura mater was cut longitudinally at the level of L7-S1 and retracted laterally. The exposed cord was then covered with a thin layer of Ringer/agar. At sites of proposed microprobe insertion a small area of agar was removed to form a pool and the underlying pia-arachnoid removed with fine sterile forceps. Such areas were then continuously irrigated with sterile Ringer solution at 37°C. Microprobes were inserted into the spinal cord with stepping motor micromanipulators. The first probe in each new area was used to obtain extracellular recordings to identify the receptive fields, to light brushing of the skin, of neurones encountered along a microprobe track.

Peripheral noxious stimulation was provided either by applying 'crocodile' clips to the pads and interdigital skin of the ipsilateral hind paw in a cycle of 3 min on, 2 min off, for the duration of the stimulus or by electrical stimulation of the ipsilateral tibial nerve (groups of three 0.5 ms square wave pulses at 330 Hz, repeated at 10 Hz, amplitude 20 V). All microprobes were inserted 3 mm into the dorsal spinal cord which, in an adult cat, places the tips in the upper ventral horn. All probes remained in the spinal cord for 30 min following insertion.

Antibody microprobes demonstrate localized binding of an endogenous ligand by their subsequent failure to bind exogenous radiolabelled ligand. Following removal from the spinal cord, microprobes were washed for 15 min in cold phosphate buffered saline containing Tween-80 (0.1%) and then incubated for 24 h at 4°C in a phosphate buffered saline/azide solution of [¹²⁵I]-neurokinin A containing 0.5% bovine serum albumin diluted to give approximately 2000 c.p.m. μ l⁻¹. Following this incubation, microprobes were again washed for 15 min in PBS-Tween with suction applied to the base of the microprobes to draw any labelled neurokinin A out of the tips. The tips were then broken off and fixed to a sheet of paper which was placed in an X-ray film cassette with a sheet of monoemulsion X-ray film (NMB-Kodak). Exposure times were typically in the order of 3–4 weeks reflecting the limited amounts of this antibody which could be bound to the probe surfaces. In each experiment 8 microprobes were used for *in vitro* tests of sensitivity. Four of these were incubated with

10⁻⁷ mol l⁻¹ neurokinin A for 30 min at 37°C prior to incubation in [¹²⁵I]-neurokinin A. The other 4 were simply incubated with the radiolabelled neuropeptide.

The resulting X-ray film images were analysed with an image analysis system employing an Imaging Technology PC Vision Plus frame grabber board operating in a DCS 286e (AT-based) computer. A television (CCD) camera was used as described previously (Hendry *et al.*, 1988) to produce a transverse integration of optical density, with a resolution of 10 μ m, along the length of each microprobe. These integrals were stored on a hard disk together with 32 coded values assigned to various experimental parameters for each microprobe. These codings allowed sorting of microprobes into groups meeting stated criteria. Images from these groups could then be averaged and the differences between control and test groups calculated and assigned levels of statistical significance. It should be emphasized that the spatial resolution of this method is 100 sites (each of 10 μ m) per mm, and that events at each site are considered independently of others in the averaging of images and the calculation of group differences and their significances.

Drug dosage and experimental regimes

The drugs used were administered via an intravenous catheter into the right cephalic vein. Morphine was administered as a 10 mg ml⁻¹ solution in physiological saline, naloxone as 1 mg ml⁻¹ in physiological saline.

As previously demonstrated (Duggan *et al.*, 1990; Hope *et al.*, 1990a) the elevation of immunoreactive neurokinin levels in the dorsal spinal cord, following a discrete stimulus, persists for up to 1 h beyond stimulation. This persistence creates difficulties when testing a compound which may block release, since a series of pre-drug measurements of stimulus-evoked release may result in a build up of immunoreactive neurokinins in the dorsal horn such that any subsequent reduction in release brought about by the drug may not be detectable. Consequently, these experiments examined the basal levels of immunoreactive neurokinins in the absence of any stimulus and then administered morphine prior to measuring stimulus-evoked release of these compounds. Several groups have shown that in the spinal cat, doses of morphine of 0.3 to 4.0 mg kg⁻¹ i.v. reduced the synaptic activation of dorsal horn neurones by peripheral noxious stimuli (Le Bars *et al.*, 1976a,b; Duggan *et al.*, 1980; Johnson & Duggan, 1981). The present experiments used morphine at levels just above the upper limit of this range (5.0 mg kg⁻¹, i.v.) since, with the protocol used, lack of effectiveness of the stimulus would be more readily detected than a partial reduction. A more significant test of the effect of morphine on immunoreactive neurokinin release came from the effects of subsequent naloxone administration. If stimulus evoked release of immunoreactive neurokinins is increased when naloxone is administered after morphine, this indicates that morphine had reduced release of these compounds. The above protocol is not optimal if morphine increases release of immunoreactive neurokinins. A series of experiments was therefore included in this study in which naloxone was administered prior to morphine in an attempt to block such an increase. Comparisons were also made with earlier studies on the effects of stimulus-evoked release of immunoreactive neurokinins in the absence of morphine (Hope *et al.*, 1990a,b).

Results

A total of 97 microprobes coated with antibodies to neurokinin A were inserted into the spinal cord. Additionally, 64 microprobes were used for concurrent *in vitro* tests. These consistently demonstrated suppression of the binding of [¹²⁵I]-neurokinin A by greater than 50% following incubation with neurokinin A 10⁻⁷ mol l⁻¹.

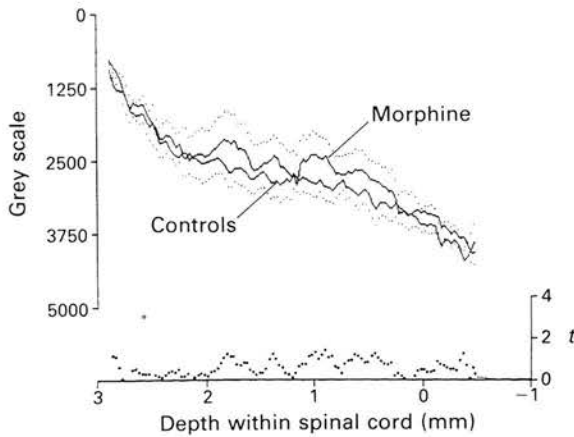


Figure 1 The effects of systemic morphine administration on resting levels of immunoreactive neurokinins in the dorsal horn. Controls: the mean image analysis of microprobes ($n = 11$) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. Morphine: the mean image analysis of microprobes ($n = 7$) inserted into the dorsal spinal cord immediately following morphine (5 mg kg^{-1} , i.v.) administration and in the absence of prior or concurrent peripheral stimulation. The analysis was performed with a resolution of 33 points per millimetre and with each group the continuous line has joined these points. The (+) s.e.mean at each point is plotted for the morphine group and the (-) s.e.mean at each point is plotted for the control group. The t values ($2t = P < 0.05$) derived from the differences between the means of the two groups are also shown.

Thirty-five probes were inserted 3 mm into the cord and left for 30 min without stimulation of either hind paw or the concurrent administration of any drug. These microprobes formed the basis for assessing possible effects of morphine or naloxone on the basal levels of immunoreactive neurokinins. The remaining 62 probes were inserted into the spinal cord in the presence of peripheral noxious stimuli and/or drug administration.

Effects of morphine and naloxone on basal levels of immunoreactive neurokinins

In four experiments, morphine was the first drug administered and in the remaining experiments naloxone was the first drug administered. Seven probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimuli, and

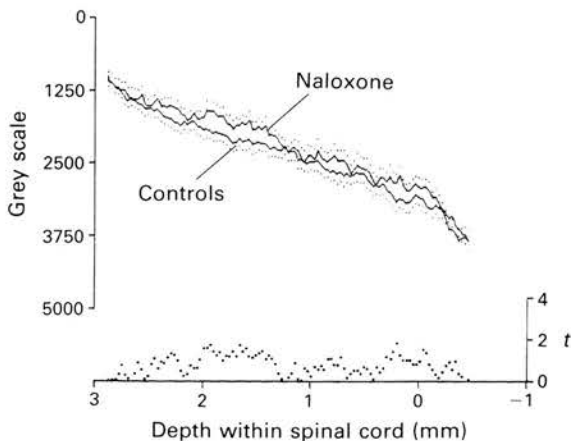


Figure 2 The effects of systemic naloxone administration on resting levels of immunoreactive neurokinins in the dorsal horn. Controls: the mean image analysis of microprobes ($n = 23$) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. Naloxone: the mean image analysis of microprobes ($n = 13$) inserted into the dorsal spinal cord immediately following naloxone (0.5 mg kg^{-1} , i.v.) administration and in the absence of prior or concurrent peripheral stimulation. S.e.means for the two groups and t values ($2t = P < 0.05$) derived from the differences of the means are shown as in Figure 1.

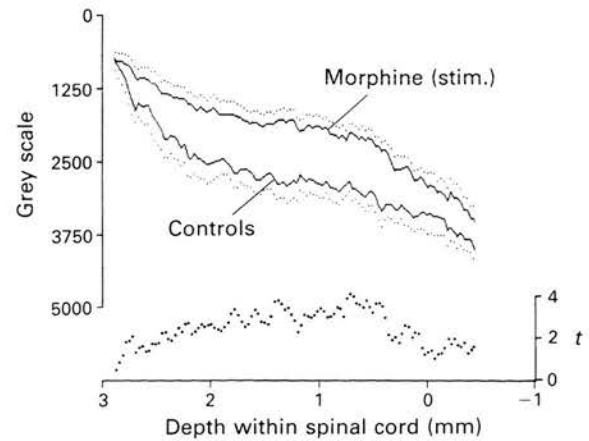


Figure 3 The effects of noxious stimulation of the ipsilateral hind paw, following systemic morphine administration on immunoreactive neurokinin levels in the dorsal horn. Controls: the mean image analysis of microprobes ($n = 11$) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. Morphine (stim): the mean image analysis of microprobes ($n = 17$) inserted into the dorsal spinal cord following morphine (5 mg kg^{-1} , i.v.) administration and during noxious stimulation of the ipsilateral hind paw. S.e.means for the two groups and t values ($2t = P < 0.05$) derived from the differences of the means are shown as in Figure 1.

within 1 min of morphine administration (5 mg kg^{-1} , i.v.). In all cases, this dose of morphine was the first drug administered. The mean image analysis of these probes is illustrated in the upper trace of Figure 1. When this group is compared to the mean image analysis of microprobes inserted in the absence of prior peripheral stimulation in the same experiments (lower trace, 'controls', Figure 1), no significant differences are present as shown in the plot of the t statistics for the differences between these groups of microprobes.

Thirteen probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimulation and within 1 min of naloxone administration (0.5 mg kg^{-1} , i.v.). No prior morphine administration had occurred. The mean image analysis of this group of microprobes and the relevant (no stimulus) controls are shown in Figure 2. The plot of the t statistics derived from the differences between these groups shows that no significant differences are present.

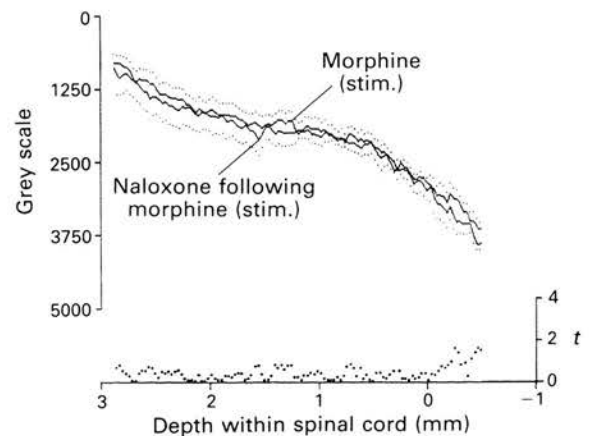


Figure 4 Effect of naloxone following morphine on noxious stimulus evoked immunoreactive neurokinin release. Morphine (stim): the mean image analysis of microprobes ($n = 17$) as described in Figure 3. Naloxone following morphine (stim): the mean image analysis of microprobes ($n = 10$) inserted into the dorsal spinal cord immediately following those illustrated in the morphine (stim) group, and after naloxone (0.5 mg kg^{-1} , i.v.) administration during noxious stimulation of the ipsilateral hind paw. S.e.means for the two groups and t values ($2t = P < 0.05$) derived from the differences of the means are shown as in Figure 1.

An additional 10 probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimulation and within 1 min of morphine administration (5 mg kg^{-1} , i.v.) but in this case morphine immediately followed naloxone (0.5 mg kg^{-1} , i.v.) administration. Again, when compared with the relevant control group no significant differences were found.

In summary, neither morphine nor naloxone, administered in the absence of peripheral noxious stimulation, produced significant changes in detectable levels of immunoreactive neurokinins in the dorsal spinal cord. A combination of these two drugs also failed to alter significantly basal levels of immunoreactive neurokinins.

Effect of morphine on stimulus-evoked immunoreactive neurokinin release

Seventeen microprobes were inserted into the dorsal spinal cord within 90 min of morphine administration (5 mg kg^{-1} , i.v.) and with concurrent noxious stimulation of the ipsilateral hind paw. Noxious pinch and electrical stimulation of unmyelinated primary afferents have previously been shown to be adequate stimuli for evoking immunoreactive neurokinin release in the dorsal spinal cord (Duggan *et al.*, 1990; Hope *et al.*, 1990a). Following morphine administration, noxious stimulus dependent immunoreactive neurokinin release was seen in the present experiments in a zone extending from the spinal cord surface to approximately 1.3 mm into the dorsal grey matter as shown in Figure 3. Although the increases in the levels of immunoreactive neurokinins in the dorsal horn produced by the stimulus appeared comparable to those produced by comparable stimuli in previous studies in which morphine had not been administered (Duggan *et al.*, 1990; Hope *et al.*, 1990a) a small inhibition of release by morphine could only be assessed by the subsequent administration of naloxone.

Eleven probes were inserted into the dorsal spinal cord within 1 min of naloxone (0.5 mg kg^{-1} , i.v.) given after morphine and during peripheral noxious stimulation. These probes were inserted after those represented in Figure 3, i.e. immediately after the period of stimulation which followed morphine administration. Figure 4 shows that naloxone had no effect on the stimulus-evoked levels of immunoreactive neurokinins in the dorsal horn which were observed following morphine. This lack of effect of naloxone suggests that the dosage of morphine used prior to naloxone had not altered the release of immunoreactive neurokinins.

Discussion

When the results of the present experiments are considered with those of Kuraishi *et al.* (1983) and Morton *et al.* (1990), there is little evidence that analgesic doses of opiates administered systemically reduce tachykinin release (either neurokinin A or substance P) from the central terminals of nociceptors. Since such results are at variance with the conclusions of experiments which have examined the effect on substance P release of topically applied opiates, these differences require comment.

Inhibition of substance P release by opiates was first shown by Jessell & Iversen (1977) in a slice preparation of rat trigeminal nucleus. The stimulus used, however, was 47 mM potassium in the perfusate. This will depolarize virtually all neural structures in the slice, and hence the source of substance P is uncertain. Furthermore, the concentration of morphine used was high (10^{-5} M). Using the push-pull cannula technique on rabbit spinal cord *in vivo*, Hirota *et al.* (1985), demonstrated a reduction in the release of substance P following noxious cutaneous stimulation with morphine (10^{-5} M) in the perfusate. Further support for the proposal that opiates impair substance P release from the central terminals of nociceptors came from the experiments of Yaksh *et al.* (1980) and Go & Yaksh (1987), in which morphine (10^{-4} M)

added to a superfusate of intact cat spinal cord *in vivo* reduced the release of substance P into the perfusate following electrical stimulation of unmyelinated primary afferents.

Mauborgne *et al.* (1987), however, found that while the μ -receptor agonist DAMGO (10^{-5} M) increased both high potassium and capsaicin induced release of substance P from dorsal horn slices, the δ -receptor agonist [D-Thr²]-Leu-enkephalin-Thr (DTLET) ($3 \times 10^{-6} \text{ M}$) reduced release in the same preparation. The κ -agonist, U50488H ($0.5\text{--}50 \times 10^{-6} \text{ M}$), did not affect release of substance P (Hamon *et al.*, 1988; Pohl *et al.*, 1989).

It should be noted that the concentrations of drugs used in all of the above studies were high and that no group has found that systemic analgesic doses of morphine reduce substance P release in the dorsal horn of spinal animals. Thus, Kuraishi *et al.* (1983), using push pull canulae in the dorsal horn of the spinal rabbit observed that systemic morphine (10 mg kg^{-1}) failed to reduce immunoreactive substance P release from noxious mechanical stimulation. Using antibody microprobes, Morton *et al.* (1990) failed to observe any effect of morphine given in two dose ranges ($1\text{--}6 \text{ mg kg}^{-1}$ and $10\text{--}20 \text{ mg kg}^{-1}$) on immunoreactive substance P release in the dorsal horn of the spinal cat following noxious mechanical or thermal cutaneous stimulation.

Since studies which have observed effects on tachykinin release have used relatively high concentrations of opiates, is the same true of experiments which have administered opiates microiontophoretically in the substantia gelatinosa and hence may have over-emphasized the importance of effects near the first central synapse of nociceptors? Although concentrations in microiontophoretic experiments are unknown, the finding that naloxone administered in the substantia gelatinosa decreased the effects of systemic morphine, 4 mg kg^{-1} , on deeper neurones (Johnson & Duggan, 1981) does support the importance of opiate receptors in the substantia gelatinosa in mediating the action of systemic morphine on spinal neurones.

When γ -aminobutyric acid acts presynaptically on the central terminals of large diameter muscle afferents, the resultant depolarization is associated with reduced transmitter release and an increased excitability when tested with an adjacent stimulating microelectrode (Curtis *et al.*, 1980). Administering opioids near the central terminals of nociceptors in the substantia gelatinosa however, has resulted in decreased electrical excitability (Sastry, 1980; Carstens *et al.*, 1987), a result not consistent with the well-established mechanism of presynaptic inhibition in the spinal cord. A different mechanism of reducing transmitter release might be if calcium entry with each invading impulse were to be reduced through a shortening of action potential duration. Such a shortening has been observed with micromolar concentrations of opioids acting on cultured dorsal root ganglion neurones (Werz & Macdonald, 1982; Shen & Crain, 1989) but lower (nanomolar) concentrations had the opposite effect (Shen & Crain, 1989). Support for a postsynaptic effect of opioids on intrinsic neurones of the substantia gelatinosa has come from Yoshimura & North (1983) who observed a hyperpolarization of such cells in a slice preparation, and Sastry & Goh (1983) who found many to be excited *in vivo* by iontophoretically administered morphine. The inconsistency in these reports however has not been explained.

Recently Lombard & Besson (1989) have tried to assess the relative importance of pre- and postsynaptic actions of morphine by recording the firing of dorsal horn neurones in decerebrate spinal rats with intact dorsal roots (and an induced peripheral arthritis) and comparable cells in non-arthritic animals with sectioned dorsal roots. Morphine (2 mg kg^{-1} , i.v.) depressed the spontaneous firing of neurones in both preparations but a greater effect was observed in the arthritic animals with intact dorsal roots. Such a result indicates an action by opiates on the pathway from primary afferent terminals to the neurones studied but cannot distinguish between effects on terminals and effects on interneurones interposed between such terminals and the neurones studied.

It has been the conclusion of studies which have observed reductions in tachykinin release with high concentrations of opiates that a presynaptic action on the central terminals of nociceptors is an important component of opiate analgesia (Yaksh & Noueihed, 1985). The present experiments considered with those of Kuraishi *et al.* (1983) and Morton *et al.* (1990) suggest that such an action does not occur significantly

with tachykinin releasing fibres in the spinal cord following clinically relevant doses of morphine.

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Lack of effect of microinjection of noradrenaline or medetomidine on stimulus-evoked release of substance P in the spinal cord of the cat: a study with antibody microprobes

¹C.W. Lang, ²P.J. Hope, ³B.D. Grubb & ⁴A.W. Duggan

Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH

1 Experiments were performed on barbiturate anaesthetized, spinalized cats to investigate the effect of microinjected noradrenaline or medetomidine on the release of immunoreactive substance P in the dorsal spinal cord following peripheral nerve stimulation. The presence of immunoreactive substance P was assessed with microprobes bearing C-terminus-directed antibodies to substance P.

2 Noradrenaline or medetomidine were microinjected into the grey matter of the spinal cord, near microprobe insertion sites, at depths of 2.5, 2.0, 1.5 and 1.0 mm below the spinal cord surface with volumes of approximately 0.125 µl and a concentration of 10⁻³M.

3 In the untreated spinal cord, electrical stimulation of the ipsilateral tibial nerve (suprathreshold for C-fibres) elicited release of immunoreactive substance P which was centred in and around lamina II. Neither noradrenaline nor medetomidine administration in the manner described produced significant alterations in this pattern of nerve stimulus-evoked release.

4 In agreement with recent ultrastructural studies these results do not support a control of substance P release by catecholamines released from sites near to the central terminals of small diameter primary afferent fibres.

Keywords: Antibody microprobes; substance P release; spinal cord; peripheral nerve stimulation; microinjection; noradrenaline; medetomidine

Introduction

Although there is considerable information on the brainstem sites of origin of descending pathways controlling spinal transmission of nociceptive information, comparatively little is known about the mechanisms operating at the spinal termination of these pathways. A wealth of evidence suggests that release of the monoamines, noradrenaline (NA) and 5-hydroxytryptamine (5-HT), mediates, in part, brainstem control of spinal nociceptive transmission. This evidence includes the effects of agonists applied either topically to the spinal cord (mouse: (Hylden & Wilcox, 1983) rat: (Camarata & Yaksh, 1985; Sullivan *et al.*, 1987; Solomon & Gebhart, 1988; Gordh *et al.*, 1989; Tseng & Tang, 1989; Danzebrink & Gebhart, 1990; Tjolsen *et al.*, 1990; Uhlen *et al.*, 1990; Fisher *et al.*, 1991; Hylden *et al.*, 1991; Takano & Yaksh, 1991) cat: (Collins *et al.*, 1984; Nakagawa *et al.*, 1990) sheep (Waterman *et al.*, 1988)) or near to single neurones in the dorsal spinal cord (Headley *et al.*, 1978; Jęftinija *et al.*, 1981; Nagy & Hunt, 1982; Curtis *et al.*, 1983; Davies & Quinlan, 1985; Fleetwood-Walker *et al.*, 1985; Zhao & Duggan, 1987) and the behavioural effects of electrical stimulation of brain stem areas exhibiting monamine containing cell bodies (rat: (Reynolds, 1969), and man (Meyerson *et al.*, 1979)). In addition there is a dense plexus of catecholamine containing nerve terminals (rat: (Schroder & Skagerberg, 1985; Rajao-fetra *et al.*, 1992), cat: (Lackner, 1980; Doyle & Maxwell, 1991a), primate (Westlund *et al.*, 1984)), and a marked density of catecholamine binding sites (rat: (Young & Kuhar, 1980), Jones *et al.*, 1982; Unnerstall *et al.*, 1984; Giron *et al.*,

1985), sheep: (Bouchenafa & Livingston, 1987) and man: (Unnerstall *et al.*, 1984)), in the superficial dorsal spinal cord, the main area of termination of small diameter primary afferent fibres.

One hypothesis for a mechanism of selective analgesia at the spinal level is a presynaptic reduction in transmitter release from primary afferent terminals of nociceptive origin. This has been investigated by a number of methods for several compounds including NA. These methods include both *in vivo* and *in vitro* transmitter release studies (Kuraishi *et al.*, 1985; Pang & Vasko, 1986; Ono *et al.*, 1991) and electrophysiological assessments of changes in the excitability of the spinal terminations of primary afferent fibres in response to compounds administered microionophoretically (Jęftinija *et al.*, 1981; Nagy & Hunt, 1982; Curtis *et al.*, 1983). Such studies however, have produced controversial results and the spinal mechanisms underlying supraspinal controls involving monoamines remain unresolved.

The most direct method of investigating a possible presynaptic control by monoamines of transmission of information conveyed by nociceptors is to measure transmitter release in the dorsal spinal cord, in response to defined peripheral noxious stimuli, before and after monoamine application close to the releasing terminals. The tachykinin neuropeptide substance P (SP) has been shown, by a variety of techniques, to be released in the spinal dorsal horn following peripheral noxious stimuli. With the antibody microprobe technique, such release in spinal cats has been shown to be centered on laminae I and II, the major sites of termination of cutaneous small diameter primary afferents (Duggan *et al.*, 1988b; 1991; 1992; Schaible *et al.*, 1992). Controls of transmitter release from the central terminals of peripheral nociceptors might, therefore, be reasonably expected to influence the stimulus-evoked release of SP in laminae I and II of spinal cats. In the present series of experiments antibody microprobes have been used to measure the release of immunoreactive SP (irSP) in the superficial dorsal horn in

Present addresses: ¹ Department of Physiology, Medical School, University of Bristol, Bristol, BS8 1TD; ² Syntex Research Centre, Research Park South, Riccarton, Edinburgh, EH14 4AP; ³ Department of Cell Physiology and Pharmacology, University of Leicester, PO Box 138, Leicester, LE1 9HN.

⁴ Author for correspondence.

response to electrical stimulation of unmyelinated primary afferent fibres. The effects of NA microinjection from a micropipette with its tip positioned in the region of the substantia gelatinosa have been evaluated in terms of any change in the subsequent pattern of stimulus evoked irSP detection. Because of existing evidence that the relevant receptors mediating the antinociceptive action of spinal NA are of the α_2 -adrenoceptor type (Fleetwood-Walker *et al.*, 1985), the effects of the highly selective α_2 -adrenoceptor agonist, medetomidine hydrochloride, were also studied.

Methods

Animal preparation

Experiments were carried out on 7 barbiturate-anaesthetized cats. These were skeletally mature animals, males and females, bred specifically for research purposes and with body weights in the range of 2.5–3 kg. Induction of anaesthesia was by intraperitoneal injection of pentobarbitone sodium (35 mg kg⁻¹) and maintenance was by a continuous intravenous infusion of the same compound (3 mg kg⁻¹ h⁻¹). All animals were artificially ventilated following neuromuscular paralysis with gallamine (4 mg kg⁻¹ h⁻¹). Gallamine was subsequently administered intermittently to allow adequate checks on the level of anaesthesia. Blood pressure was monitored via a cannulated carotid artery and end-tidal CO₂ levels were continually monitored and maintained at 4%.

The lumbar spinal cord was exposed by removal of the overlying bony laminae and the spinal cord was then transected at the thoraco-lumbar junction following intraspinal injection of 0.1 ml of a 2% lignocaine solution. The lumbar dura mater was cut longitudinally and retracted laterally. A thin layer of Ringer/agar was then placed over the dorsal surface of the exposed spinal cord. At sites of proposed microprobe insertion an area of agar and a small part of the underlying pia-arachnoid were removed with sterile fine forceps. The area of the spinal cord exposed in this way was then irrigated with a sterile Ringer solution held at 37°C by a heat exchanger jacket around the delivery tube. Following the induction of anaesthesia, cats received two intramuscular injection of ampicillin, 25 mg kg⁻¹, 10 h apart. When measuring neuropeptide release by any method it is important to have near sterile conditions (Duggan, 1992).

Although irSP release in the superficial dorsal horn is evoked by a variety of noxious peripheral stimuli (Duggan *et al.*, 1988b), it is difficult to deliver such stimuli repeatedly without damaging peripheral tissues and thus altering the firing of peripheral nociceptors to successive stimuli. For this reason, electrical stimulation of unmyelinated primary afferent fibres in the tibial nerve was used to elicit spinal release of irSP in the present series of experiments. The right and left tibial nerves were exposed in the lower limb, immersed in liquid paraffin and mounted on platinum stimulating electrodes. Stimulation was with square wave pulses, 0.3 ms duration, with an amplitude of at least 80 times the threshold stimulus needed to produce a short latency field potential as measured with a recording electrode placed in the upper dorsal horn. Stimulus frequency was 20 Hz and stimulation duration was 10 min in every case.

Microprobe preparation

Antibody microprobes were prepared as previously described (Duggan *et al.*, 1988a; Duggan, 1992). Briefly, fine glass micropipettes, heat sealed at both ends, were incubated in a 10% solution of aminopropyltriethoxysilane in toluene. This produced a siloxane polymer layer on the outer surfaces of the microprobes and glutaraldehyde was then used to immobilize protein A (Sigma) to this polymer. Protein A then bound immunoglobulins present in a polyclonal antiserum

containing antibodies directed at the C-terminus of SP (Peninsula Laboratories). These antibodies bind SP and fragments of SP containing at least the last five amino acid residues; data from the manufacturer indicates negligible cross-reactivity with either neurokinin A or neurokinin B.

Microprobes were inserted into the spinal cord, in pairs, with stepping motor micromanipulators. Prior to insertion, microprobes had both ends removed to allow filling with a solution of pontamine sky blue to aid visualization of the tips. With the first probe introduced into a particular area of the spinal cord, it was usual to obtain extracellular recordings during introduction into the spinal cord in order to confirm that fibres in the ipsilateral tibial nerve did project to that area. All microprobes were inserted to a depth of 4 mm which, in the lumbar spinal cord of the adult cat, places the tip in the ventral horn. Microprobes were left *in situ* for 10 min following insertion into the cord, during which time either (a) no peripheral stimulus was applied to allow detection of basal irSP levels or (b) the ipsilateral tibial nerve was stimulated as previously described, with or without prior drug application (see results). Insertions of consecutive pairs of antibody microprobes, in the same area of the spinal cord, were at intervals of at least 25 min.

The antibody microprobe technique detects bound endogenous ligand by the subsequent failure of binding of exogenous radiolabelled ligand. Thus, following removal from the spinal cord, microprobes were washed for 15 min in a cold solution of phosphate-buffered saline (PBS) containing Tween (0.1%) and then incubated for 24 h at 6°C in a PBS-azide solution of (Bolton-Hunter) ¹²⁵I-radiolabelled SP (Amersham) containing 0.5% bovine serum albumin. This solution was diluted to give approximately 2000 counts min⁻¹ µl⁻¹. After this incubation, microprobes were again washed for 15 min in cold PBS-Tween, while continually drawing the solution through the tips to remove any radiolabelled SP from the inside. The tips were then carefully broken off about 1 cm back and glued to a sheet of paper which was placed in an X-ray film cassette with a sheet of monoemulsion film (Kodak NMB).

The resulting X-ray film images of microprobes were analysed with an image analysis system employing an Imaging Technology PC Vision Plus frame grabber board operating in a Data Control Systems 286e (AT based) computer. A CCD camera scanned each image and, as described previously (Hendry *et al.*, 1988), after background subtraction in a transverse integration of the optical density of the image of each microprobe was executed at defined intervals. With the magnification used and the resolution of the image analysis system (512 by 512 locations per frame) this corresponds to a 10 µm interval for transverse integrations. The resultant integrals were stored on a hard disk record which included 40 coded values for each individual image relevant to the experimental conditions surrounding it. An analysis programme subsequently obtained groups of microprobes which met stated criteria and obtained the mean image analysis of each group. This mean image analysis was plotted with respect to distance from spinal cord surface (see Figures 1a, 2 and 3a). In addition, differences between the mean image analyses of control and experimental groups were obtained, *t* statistics were then calculated and a probability of significance assigned to the difference in the mean grey scale values at each analysis point (see Figures 1b and 3b). It is important to emphasize the spatial resolution of this technique. For microprobes inserted into the spinal cord events are examined optically at 100 sites per mm. Since such a resolution exceeds the biological resolution of the microprobe technique, the mean of 3 successive sites was calculated and stored giving an approximate resolution of 33 sites per mm.

During *in vitro* tests of antibody microprobes, [¹²⁵I]-SP was bound to a representative group of probes. At least 50% suppression of this binding of radioactive SP was achieved by prior incubation of the microprobes in a solution of 10⁻⁷ M unlabelled SP for 30 min at 37°C.

Microinjection of drug solutions

NA and medetomidine (a gift from Farnos) solutions (10^{-3} M in sterile Ringer) were microinjected into the dorsal horn from micropipettes (tip diameter 20–40 μ m) positioned with a micromanipulator to the appropriate regions and at depths of 1.0, 1.5, 2.0, and 2.5 mm below the dorsal spinal cord surface. Although in the cat, the peak release of irSP evoked by tibial nerve stimulation is in the superficial dorsal horn, 1.1 mm below the cord surface, (Duggan *et al.*, 1988b; 1991; 1992; Schaible *et al.*, 1992), there is significant release from approximately 0.5 to 1.0 mm deep in this area. Hence the microinjections were performed at sites deep in the region of the substantia gelatinosa. The total drug volume was 0.5 μ l, i.e. 0.125 μ l at each site. Previous experiments (Duggan *et al.*, 1991; 1992; Schaible *et al.*, 1992) have shown that microinjections of Ringer solution (or of PBS), in comparable or larger volumes to those used in the present study, have no effect on the stimulus-evoked release of irSP in this region of the spinal cord.

The timing of microinjections in relation to the application of stimuli evoking release of irSP is important in experiments of this type for several reasons. Firstly, if a compound is inactivated quickly, the microinjection may need to be performed whilst a microprobe is already *in situ* and a stimulus being delivered. Previous experiments with neuropeptide (NPY) microinjection (Duggan *et al.*, 1991), inhibitors of endopeptidase 24.11 (Duggan *et al.*, 1992) and calcitonin gene-related peptide (CGRP) (Schaible *et al.*, 1992) showed that this was not necessary with these compounds. Secondly, the injected compound may need to diffuse within the spinal cord for an effect of irSP release to be detected; this implies a need to inject some time prior to testing for inhibition of release. In the present experiments injections of NA or medetomidine were performed after control microprobes (either no stimulus or nerve stimulus controls) had been removed from the cord and these microinjections were then given gradually over a 5 min period. Subsequent pairs of microprobes were then inserted as rapidly as possible into the cord and were typically *in situ* 1–2 min after cessation of drug microinjection. Drug microinjections and subsequent microprobe insertions during nerve stimulation were always at least 25 min after the preceding period of ipsilateral tibial nerve stimulation.

Both NA and medetomidine were microinjected using 10^{-3} M solutions and in volumes of approximately 0.125 μ l at each site. These volumes correspond to spheres with a radius of approximately 340 μ m and in the absence of a removal mechanism, such as neuronal uptake, a 10 fold reduction in concentration (to 10^{-4} M) will occur with an expansion of this sphere, by diffusion, to a radius of 736 μ m. Drug absorption into local blood vessels will reduce the concentration still further, but as the starting concentrations for both NA and medetomidine were relatively high and the drugs were also administered close to the major zone of primary afferent fibre termination, the concentrations of drug used should have been sufficient to reveal any action at such sites. This methodology has successfully altered the pattern of release of SP following microinjection of NPY or CGRP (Duggan *et al.*, 1991; Schaible *et al.*, 1992).

Results

Stimulus-evoked release of immunoreactive substance P

The mean image analyses of 13 microprobes inserted 4 mm into the spinal cord and left for 10 min in the absence of peripheral nerve stimulation, and 24 microprobes inserted for the same time and to the same depth but during electrical stimulation of myelinated and unmyelinated primary afferents of the ipsilateral tibial nerve are illustrated in Figure 1a. These microprobes are derived from the same experiments.

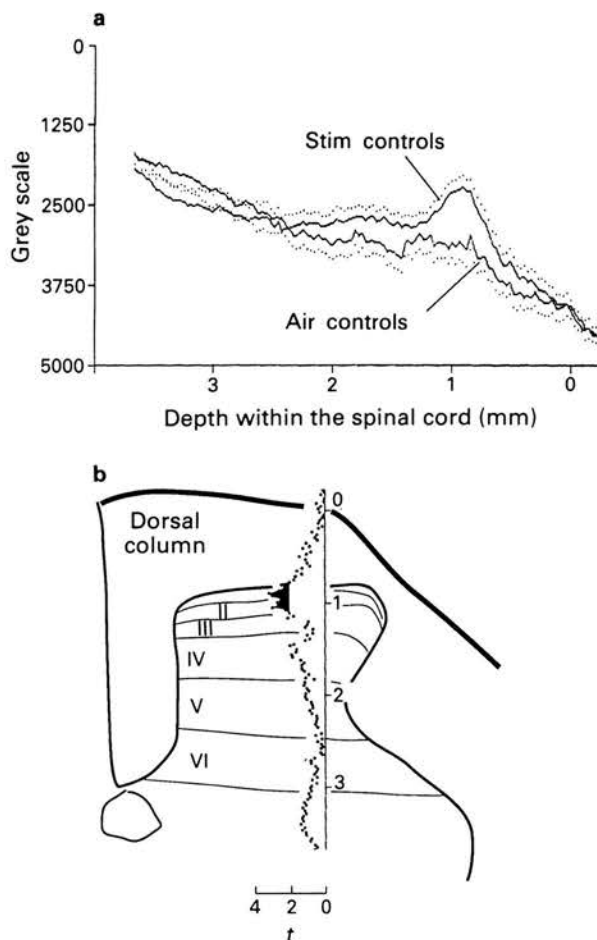


Figure 1 Nerve stimulus-evoked release of immunoreactive-substance P in the superficial dorsal horn. (a) The mean image analysis of two groups of microprobes are plotted: those which remained in the spinal cord for 10 min and no peripheral stimulus was applied (air controls, $n = 13$) and those present in the spinal cord during electrical stimulation of myelinated and unmyelinated afferents of the ipsilateral tibial nerve (skin controls, $n = 24$). The mean grey scale of microprobes was calculated in 30 μ m intervals and a line joins these points. At each point the s.e.mean (+ for Stim controls, - for air controls) is plotted. (b) A plot of the *t* statistics derived from the differences of the means from the two groups of microprobes shown in (a). This plot is superimposed on a diagram of a cross section of the lumbar spinal cord of the cat and the hatched area indicates where these differences are significant with a probability of < 0.05 .

Significant differences between these mean image analyses occur from 0.7 to 1.2 mm from the dorsal surface of the spinal cord, and there is also a smaller zone of significance approximately 1.5 mm below the cord surface. The distribution of significant differences between the two groups is illustrated in Figure 1b. The maximal difference occurs at a depth of 1.0 mm below the spinal cord surface. This site of maximal release approximates to the substantia gelatinosa and lamina I of the dorsal grey matter and agrees with the findings of previous studies (Duggan *et al.*, 1988b; 1991; 1992; Schaible *et al.*, 1992). When dye is ejected from the tip of a single microprobe and its position in the spinal cord subsequently determined in a spinal cord section, then sites of neuropeptide release can be inferred with great accuracy. With mean image analyses, as presented here, the location of sites of release is less precise. With microprobes inserted a fixed distance into the spinal cord (4 mm in this series of experiments) the relationship of spinal laminae to distance from the tip will vary between individual animals, and also with distance from the midline. When encoding the information describing the position of a microprobe, a correction

factor is included when appropriate, but the relative broadness of the sites of release of irSP results in part from these anatomical differences.

Microprobes present in the spinal cord following microinjection of noradrenaline

The mean image analyses of 38 microprobes inserted 4 mm into the spinal cord for 10 min with concurrent electrical stimulation of myelinated and unmyelinated afferents of the ipsilateral tibial nerve (stim. controls) and 35 microprobes inserted under the same conditions but following the injection of 10^{-3} M NA at depths of 1.0, 1.5, 2.0 and 2.5 mm below cord surface in the area of subsequent microprobe insertions (post NA) are illustrated in Figure 2. The two groups of microprobes are derived from the same experiments. The two mean image analyses are virtually identical and at no points do the differences between them approach statistical significance (at $P < 0.05$).

Microprobes present in the spinal cord following microinjection of medetomidine

The mean image analyses of 31 microprobes inserted 4 mm into the spinal cord for 10 min with concurrent electrical stimulation of the myelinated and unmyelinated afferents of the ipsilateral tibial nerve (stim. controls) and 20 microprobes inserted under the same conditions but following the microinjection of 10^{-3} M medetomidine at depths of 1.0, 1.5, 2.0 and 2.5 mm below the spinal cord surface in the area of subsequent microprobe insertion (post Med) are illustrated in Figure 3a. These two groups are again derived from the same experiments, i.e. the 'Stim control' groups illustrated in Figures 2 and 3a do not represent the same microprobes. Although the post medetomidine group is displaced above the control group at sites deep to the superficial dorsal horn, (suggestive of increased release) there are no statistically significant differences between the two groups within the spinal cord as shown in Figure 3b. A small area of increased irSP detection following medetomidine microinjection is evident at the cord surface. This has been an observation in previous studies with microprobes and has been related to the late development of inflammation at the sites of removal of pia mater at the cord surface (Duggan *et al.*, 1988b) in some experiments. Hence it is more likely to be seen in the post injection than pre injection microprobes.

Thus, neither 10^{-3} M NA nor 10^{-3} M medetomidine, microinjected directly into the dorsal spinal cord, had any

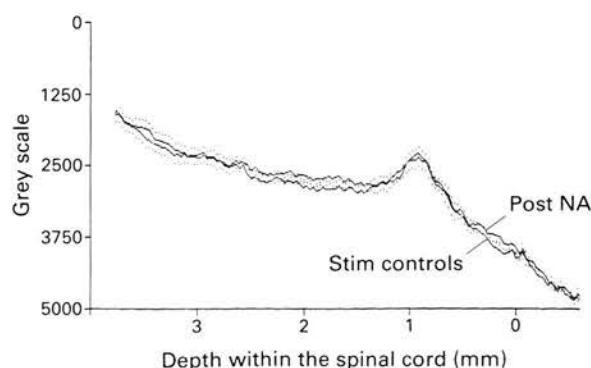


Figure 2 Lack of effect of microinjection of noradrenaline (NA) into the dorsal horn on the nerve-stimulus-evoked release of immunoreactive-substance P. The mean image analysis of two groups of microprobes are plotted: those present in the spinal cord for 10 min during electrical stimulation of myelinated and unmyelinated afferents of the ipsilateral tibial nerve (Stim controls, $n = 38$) and those with comparable stimulation but inserted after microinjection of noradrenaline (10^{-3} M) into the dorsal horn (post NA, $n = 35$). The format of plotting is similar to that of Figure 1a.

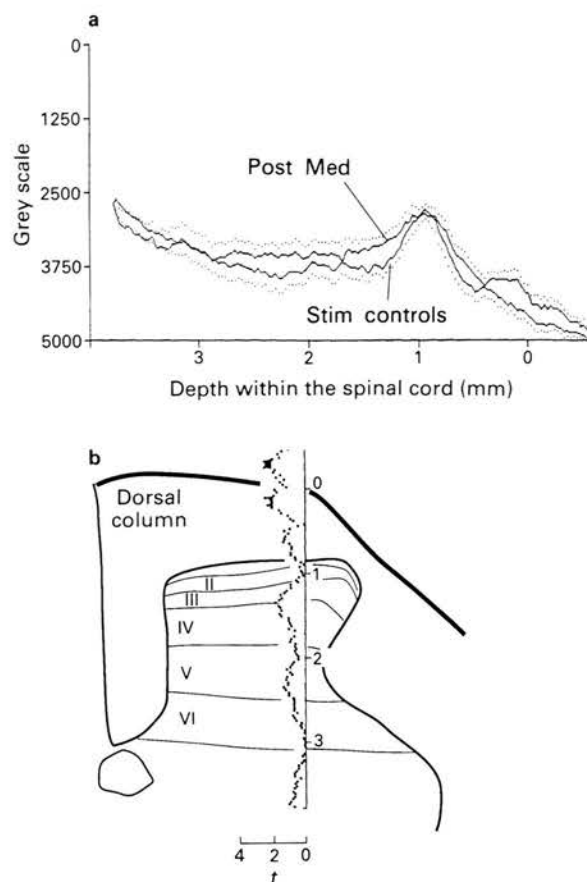


Figure 3 Lack of effect of microinjection of medetomidine into the dorsal horn on the nerve stimulus-evoked release of immunoreactive-substance P. (a) The mean image analysis of microprobes present in the spinal cord for 10 min during electrical stimulation of myelinated and unmyelinated primary afferents of the ipsilateral tibial nerve (Stim controls, $n = 31$) and those with comparable stimulation but inserted after microinjection of medetomidine (10^{-3} M) into the dorsal horn (post Med, $n = 20$). (b) A plot of the t -statistics derived from the differences of means of the two groups shown in (a) is superimposed on a diagram of the lumbar spinal cord of the cat. The hatched area indicates significant differences with a probability of < 0.05 .

significant effect on the subsequent nerve stimulus-evoked release of irSP.

Discussion

The present results are part of an extended study of possible presynaptic controls of tachykinin release from the central terminals of nociceptors. Presynaptic inhibition of transmitter release from such terminals has been proposed as important in several mechanisms of analgesia, including that resulting from impulses arriving in large diameter afferents (Melzack & Wall, 1965) and that produced by electrical stimulation of certain brainstem regions (Reynolds, 1969; Meyerson *et al.*, 1979).

There is evidence that, in acutely spinalized cats, the irSP detected by microprobes and localized to the region of the substantia gelatinosa following peripheral nerve stimulation, is largely released from the central terminals of primary afferent fibres. The substantia gelatinosa is the major site of termination of cutaneous unmyelinated primary afferent fibres (Sugiura *et al.*, 1986), and a proportion of these are known to contain substance P (Nagy & Hunt, 1982; Price, 1985). A contribution from intrinsic substance P-containing spinal neurones is possible, but in the spinalized animal

release from fibres of supraspinal origin cannot have occurred. Thus physiological controls acting on the terminals of unmyelinated primary afferents should be revealed by this methodology, provided the compounds are administered in a relevant manner.

Although microinjection of NA directly into the substantia gelatinosa had no effect on the stimulus-evoked release of irSP, administration of drugs in this manner has previously been shown to be an effective way of modulating stimulus-evoked irSP release. Microinjection of NPY was shown to reduce irSP detection following peripheral nerve stimulation (Duggan *et al.*, 1991). Conversely, microinjection of CGRP or of a mixture of the peptidase inhibitor drugs, ketanorphan and enalaprilat, enhanced post-stimulus detection of irSP by antibody microprobes (Duggan *et al.*, 1992; Schaible *et al.*, 1992). Microinjections of comparable volumes of Ringer or PBS solutions in these studies were without effect. NA may, following microinjection, have been subject to rapid re-uptake and degradation in the spinal cord. Medetomidine, a synthetic compound, does not appear to be subject to any re-uptake mechanism (R. Virtanen, Farnos, personal communication). Following systemic administration, medetomidine is metabolized by hydroxylation in the liver (Salonen & Eloranta, 1990) but little is known of its elimination from the central nervous system.

The current observation that NA was ineffective in reducing noxious stimulus-evoked irSP release disagrees with earlier studies of catecholamines and SP release from the spinal cord. Ono *et al.* (1991) demonstrated that clonidine (10^{-5} M) and tizanidine (10^{-4} M), both α_2 -adrenoceptor agonist drugs, reduced the veratridine-induced release of SP from slices of rat spinal cord *in vitro*. This effect was blocked by yohimbine (10^{-5} M) and also by prazosin (10^{-5} M), suggesting the involvement of α_{2B} -adrenoceptors. Pang & Vasko (1986), reported that NA (10^{-5} M) inhibited the high potassium-evoked release of SP from a rat spinal cord slice. Veratridine application or simple elevation of extracellular potassium levels do not selectively activate primary afferent fibres and perfusion of cord slices allows no spatial resolution of the source of neurotransmitter release. Thus SP will probably be released from a host of structures in these experiments, including fibres of supraspinal/proprio-spinal origin and intrinsic neurones of the slice as well as primary afferent terminals. Thus the action of α_2 -agonists cannot necessarily be related to an action on primary afferent terminals.

More directly comparable to the present study are the experiments of Kuraishi *et al.* (1985) who reported that NA (10^{-5} M) added to the perfusate of a push-pull cannula inserted into the upper dorsal horn of rabbit spinal cord *in vivo* reduced the release of irSP in response to a peripheral noxious mechanical stimulus. The cannulae used were of 600 μ m outer diameter and the flow rate was 50 μ l min $^{-1}$ (0.83 μ l s $^{-1}$). Although it is uncertain how much of the perfusate passing through a push-pull cannula escapes into the surrounding tissue, 0.83 μ l s $^{-1}$ is a relatively large flow rate in relationship to the dimensions of the dorsal horn of the rabbit. The distance from the dorsal surface of the spinal cord to lamina I of the grey matter is approximately 1 mm and as 1 μ l represents 1 mm 3 , it is probable that administration of NA in this way results in activation of adrenoceptors over a very wide area of the spinal grey and white matter.

Indeed, an important distinction between the present study and those in which effects on SP release have been observed is the method of drug administration. In our experiments, NA and medetomidine microinjections were restricted to the dorsal horn and the volumes were relatively small.

Experiments in which large volumes of drug containing solutions are administered will access the intraspinal preterminal regions of primary afferents running rostrally and caudally in the white matter and this could be a cause of reduced stimulus-evoked release of SP which is not related to a physiological mechanism. There is good evidence for 5-HT receptors on the peripheral branches of unmyelinated pri-

mary afferents (Neto, 1978) and for γ -aminobutyric acid (GABA) receptors on undefined peripheral nerve fibres (Sakatani *et al.*, 1991). A recent study (Sakatani *et al.*, 1993) found that GABA, 10^{-4} M, applied to the hemisectioned spinal cord of the neonatal rat, reduced the amplitude of the compound action potential resulting from propagation of impulses along dorsal column fibres. This was a receptor-mediated and not a local anaesthetic type of effect since it was reversed by bicuculline. Such a block of conduction in some fibres would reduce transmitter release at the relevant terminals. Thus caution is needed in interpreting the results of experiments in which adrenoceptor agonists are administered either diffusely to a slice preparation or intraspinally in large volumes as necessarily revealing a physiological control of SP released from the central terminals of nociceptors. Although we do not know the precise areas at which pharmacologically active doses of these compounds were present in our experiments, it is probable that restricting administration to areas near the central terminals of nociceptors is more likely to study controls acting at those terminals.

Ultrastructural studies have also failed to find evidence for a synaptic control by catecholamines of transmitter release from the central terminals of nociceptors. In both the rat (Sugiura *et al.*, 1986) and cat (Doyle & Maxwell, 1991a,b; 1993a) axo-axonic contacts containing dopamine- β -hydroxylase or tyrosine hydroxylase in the presynaptic element were not identified in the region of the substantia gelatinosa, the major site of termination of small diameter primary afferent fibres. Axo-dendritic and axo-somatic contacts were readily identified in the same area. These contacts have also been observed with identified lamina I spinothalamic tract cells of the monkey (Price, 1985). It is interesting to note that previous results from our laboratory which provided evidence for a control by NPY of the spinal release of SP following activation of peripheral nociceptors (Duggan *et al.*, 1991) have been supported by recent ultrastructural studies (Doyle & Maxwell, 1993b).

Electrophysiological studies of the effects of NA and 5-HT on the electrical excitability of the central terminals of small diameter primary afferents have given results allowing for more than one interpretation, and have not helped resolve the issue of whether presynaptic α_2 -adrenoceptors are involved in physiological controls at such sites. With large diameter primary afferents, presynaptic inhibition by GABA is mediated by a terminal depolarization detected as an increased excitability to stimulation by an adjacent microelectrode (Curtis *et al.*, 1977). Both Carstens *et al.* (1982) and Jeftinija *et al.* (1981) found that microiontophoretically administered monoamines increased the electrical threshold of the central terminals of small diameter afferents. Curtis *et al.* (1983) suggested that this was an artifact due to an altered current distribution during electrical stimulation as a result of glial uptake of administered NA or 5-HT, rather than a novel mechanism of presynaptic inhibition.

The present results do not support the proposal that there is a physiological control of SP release from the central terminals of nociceptors mediated by α_2 -adrenoceptors. This is in accord with ultrastructural and electrophysiological observations. It is more probable that the potent effects of α_2 -adrenoceptor agonists in suppressing spinal transmission of nociceptive information result from postsynaptic actions on neurones of the spinal cord. We believe that experiments purporting to support presynaptic control mechanisms are difficult to interpret both in terms of the source of the released SP by the stimuli used, and/or the sites accessed by the method of drug administration.

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